# On the Role of TNFα in CD40 Mediated Sickness Behaviour Syndrome

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# 1. ZUSAMMENFASSUNG

Das sogenannte Krankheitsgefühl Syndrom (engl. sickness behavior syndrome, SBS) beschreibt die Gesamtheit der Adaptierung des Verhaltens und der Physiologie von viralen oder bakteriellen Infektionen der Betroffenen. Es umfasst uns allen begegnete Symptome, wie Fieber, Appetitlosigkeit, Verstimmung, Müdigkeit, Lethargie und verminderte soziale Interaktion. Nicht bewiesen aber vermutet wird, dass das SBS eine Strategie erfüllt, um die Infektionen besser zu bewältigen und das Ansteckungsrisiko zwischen Individuen zu minimieren. Bei chronischen Entzündungskrankheiten und in Autoimmunkrankheiten, wie zum Beispiel der Multiplen Sklerose, rheumatoiden Arthritis und dem systemischen Lupus erythematodes, ist das SBS eher maladaptiv und beeinträchtigt die Lebensqualität der Betroffenen sehr stark.

Eine Vielzahl von wissenschaftlichen Untersuchungen zeigt, dass das SBS durch Aktivierung des Immunsystems von Zytokinen hervorgerufen und aufrechterhalten wird. Am häufigsten diskutiert sind dies, der Tumor Nekrose Faktor (TNF), Interleukin-1 (IL-1) und Interleukin-6 (IL-6), für welche gezeigt ist, dass sie Zentren im Hypothalamus erreichen oder alternativ über den Nervus Vagus die Funktion des neuronalen Netzwerkes beeinflussen.

Die molekularen Mechanismen welche zu den von Zytokinen bewirkten Veränderungen der Physologie, des Metabolismus und des Verhaltens führen, sind noch wenig untersucht.

Die Auswirkungen des zirkadianen Systems auf unsere Gesundheit rücken immer mehr in allgemeines Interesse. Es wird vermutet, dass zirkadianische Dysfunktionen zu einem breiten Spektrum von Krankheiten beitragen, wie zum Beispiel Insomnie und Depressionen.

Eine steigende Anzahl von Studien zeigt, dass das Immun- und das zirkadianische System sich gegenseitig regulieren könnten. Vor kurzem wurde berichtet, dass das Zytokin TNF in der Lage ist die Expression der Clock Gene *Period*, *Rev-Erbα* und der Clock-kontrollierten Gene zu beeinflussen, was auf ein direktes Zusammenspiel zwischen den Clock Proteinen und dem Immunsystem hinweist.

Hier wird gezeigt, dass die Behandlung mit agonistischen anti-CD40 Antikörpern ein geeignetes Model darstellt um SBS, welches mit Autoimmunkrankheiten assoziiert ist, zu erforschen. CD40 ist ein vor allem auf Antigen präsentierenden Zellen (Dendritischen Zellen und Makrophagen) sowie B-Zellen exprimiert. Es wird durch die von T-lymphozyten gebildeten CD40 Liganden aktiviert. Hemmung der CD40 Aktivierung führt zu verminderter Ausprägung von Autoimmuerkrankungen in Tiermodellen. Wir fanden, dass in Mäusen die Verabreichung von agonistischen anti-CD40 Antikörpern zu einer ausgesprochen Reduktion

der lokomotorischen Aktivität und zu einer Zunahme der ruhenden Periode in der Nacht führt, während die Tiere normalerweise aktiv sind. Dieser Effekt wurde auch in Mäuse welche kein IL-6 oder kein IL-1R1 exprimieren beobachtet. Dies ganz im Gegensatz zu Mäusen, welche keinen TNFR1 exprimieren. Diese Untersuchungen belegen, dass die TNF-TNF Rezeptor 1 Interaktion für das Auftreten von SBS nach CD40 Aktivierung unabdingbar ist. Dafür sprechen auch Untersuchungen von Mäusen, welche kein MyD88 – ein intrazelluläres Adaptorprotein das mit Pathogenen Erkennungsstrukturen und IL-1R1 kommuniziert, exprimieren. $MyD88^{-/-}$ Mäuse sind genauso wie $TNFR1^{-/-}$ resistent gegenüber Behandlung mit agonistischen anti-CD40 Antikörpern, doch diese Mäuse bilden darauf nur vermindert TNF im Serum. Eine Verabreichung von rekombinanten TNF führt in diesen Mäusen ebenfalls zu SBS.

Wie schon nach TNF Behandlung von Mäusen gezeigt, war die Amplitude der Expression von E-Box kontrollierten Clock Genen in der Leber von agonistischen anti-CD40 Antikörpern behadelten Tieren abgeschwächt. Dieser Effekt auf die Expression der Clock Genen, ist ebenso wie die Reduktion der Aktivität der Tiere, von TNFR1 abhängig, nicht aber von IL-6 oder IL-1R1. Das Ausmass der Aktivitätverminderung korreliert mit der Intensität der Repression von *Dbp* in allen untersuchten Genotypen.

Obwohl der molekulare Mechanismus der hier geschilderten Ergebnisse noch geklärt werden muss, zeigen diese Ergebnisse, dass das Immunsystem über TNFR1 die zirkadiane Uhr beeinflusst und dadurch zur Entwicklung des SBS führt.

## 2. SUMMARY

The sickness behaviour syndrome (SBS) describes the collective behavioural and physiological adaptations of the host to viral and bacterial infections. It comprises well-known symptoms, such as fever, loss of appetite, depressive mood, fatigue, reduced social interaction and lethargy. It is considered as a strategy to better cope with infections, to facilitate recovery, and to decrease the risk of disease transmission among individuals. The behavioural changes observed in sick individuals may be indeed advantageous to the infected host, in line with experiments showing that sickness behaviour is a motivational state rather than just weakness alone. However, in the course of autoimmune diseases, including multiple sclerosis, lupus erythematosus, and rheumatoid arthritis, SBS seems to be rather maladaptive, and often represents the most debilitating effect compromising the quality of life of the affected individuals. Many lines of evidence indicate that SBS is induced and maintained by cytokines released by activated immune cells. Among these, tumour necrosis factor-alpha (TNF), interleukin-1β (IL-1), and interleukin-6 (IL-6) are the most potent mediators, signalling to the brain via humoral and neural pathways. However, their functional hierarchy and the molecular mechanisms underlying the physiological, metabolic, and behavioural action of these cytokines have not been investigated yet.

The impact of circadian timing on our health is gaining increasing attention, and circadian dysfunction is thought to contribute to the incidence of a wide range of diseases, including sleep disorders and depression. An increasing number of studies indicate that the immune system and the circadian timekeeping system may mutually regulate each other. Previous evidence suggests that the cytokine TNF can modulate the expression of clock genes *Bmal1*, *Period*, *Rev-Erbα* and of the clock-controlled genes, suggesting a directly link between core clock proteins and inflammatory pathways.

Here, we report that treatment with CD40 agonists represents a suitable model for studying sickness behaviour syndrome associated with autoimmune diseases. The CD40-receptor is expressed on the cell surface of B-lymphocytes and of antigen presenting cells, including macrophages and dendritic cells. These cells become activated by T-cells, which express CD40 ligand (CD40L). Blocking of CD40 activation leads to reduced severity of autoimmune diseases in animal models. In this model, TNF is likely the main mediator of the behavioural changes observed. We found that injection of CD40 agonists leads to a pronounced impairment of locomotor activity and increased rest periods in mice at night, the time period when the animals are usually active. This effect was still observed in IL-6 and IL-1R deficient

mice, but was not seen in mice lacking TNFR1. Moreover, TNF injections were sufficient to induce SBS in MyD88$^{-/-}$ mice, although these animals were resistant to SBS mediated by CD40 agonists. MyD88 is an intracellular adaptor protein used by all Toll-like receptors (except TLR3) and IL1R1.

As reported for TNF, the amplitude of the expression of E-box bearing clock genes was reduced in the liver of CD40 treated animals. The impairment of locomotor activity as well as the dysegulation of clock gene expression depends on TNFR1, but not on IL-6 or IL-1R1 signalling. The degree of locomotor activity impairment correlates with the intensity of *Dbp* repression and *Bmal1* induction in all genotypes analyzed.

Although a molecular mechanism still needs to be clarified, these results indicate that the immune system is indeed able to influence the circadian clock. Interference of the immune system with the circadian clock may represent a possible mechanism underlying the development of sickness behaviour syndrome.

# 3. INTRODUCTION

## 3.1 Cytokine-induced sickness behaviour

### 3.1.1 The sickness behaviour syndrome

Everybody has experienced the feeling of being sick after a bacterial or viral infection. Typical symptoms of sickness include fever, loss of appetite, weight loss, sleepiness, listlessness and fatigue. They are collectively referred to as sickness behaviour syndrome (SBS). Initially, it was thought that the behaviour of a sick subject was due to physical weakness that resulted from diverting energy to the body processes needed to fight infection. Now, however, it is known that sickness represents a complex adaptive response to infection or injury required to improve chances of survival and decrease the risk of disease transmission among individuals [1].

The first evidence for the benefits of fever in fighting disease came from experiments by Kluger and colleagues. They showed that survival of lizards infected with a pathogen was enhanced if the animals were allowed to develop fever [2]. Few years later, in a similar experiment performed in rabbits, treatment with an antipyretic agent increased mortality in *P. multocida* infected animals [3]. In 1979, Murray et al. showed that overriding the anorexia of mice infected with *Listeria monocytogenes* by force-feeding, both accelerated the course of infection and increased the probability of death [4]. Thus, the development of SBS seems to offer survival and healing advantages to many animal species. SBS, however, also represent the most disabling consequences for patients with autoimmune diseases [5-8], and have stronger debilitating effects on the quality of life of the affected subject than the pain or disease associated symptoms themselves. Moreover, increasing evidence suggest that inflammation increases the risk of occurrence of major depressive disorders, and pro-inflammatory cytokines have been implicated as contributing factors [9]. Common to all patients suffering from conditions associated with fatigue, due to both infectious and autoimmune diseases, are altered levels of cytokines in the blood.

Already in 1960 it was known that symptoms of sickness were due to blood-derived factors acting on the brain [10]. In the 1980s, when IL-1 was first isolated and later on with the discovery that TNF induces endotoxic shock, it became clear that these factors were cytokines, produced by immune cells in response to endotoxin.

Besides being the major signalling molecules of the immune system, cytokines can also act as potent neuromodulators. This may help to explain the cytokine-induced CNS derived behavioural effects of SBS. The immune system has long been regarded as an immune privileged site. However, the last decades provided strong evidence that the central nervous system and the immune system are able to communicate with each other, the one system being modulated by the other resulting in bidirectional neuroimmune interactions [11]. There are many cytokines that can affect brain function and therefore behaviour, but TNF, IL-1, IL-6 are the cytokines which have been consistently associated with SBS [12]. It is interesting to note that systemic or central administration of IL-1, TNF or IL-6 into mice induces the full spectrum of behavioural signs of sickness, suggesting that these cytokines are involved in the CNS response [9]. Cytokines never act alone, but rather in a complex network in which they can induce expression of each other and regulate the expression of their own receptors [12]. Although the importance of these cytokines in mediating sickness behaviour has been appreciated, there is still limited knowledge about the hierarchy of the involved cytokines, their site of action and the interplay between the different modulators involved in a complex immune response regulating sickness behaviour.

### 3.1.2 The immune system regulates the production of cytokines

Dendritic cells and macrophages, representing the innate immune system, mediate the first step of the defence against harmful pathogens. These cells recognize pathogens or endogenous danger signals, released upon cellular damage or stress, via pattern-recognition receptors (PRRs). The best known of these are Toll-like receptors (TLR), but many other receptors are also involved. PRRs bind to conserved microbial components, known as pathogen-associated molecular patterns (PAMPs), or endogenous alarmins. Ligand-bound PRRs alert the host of danger by activating the immune system, leading to the production of pro-inflammatory cytokines by immune cells [13].

Cytokines have a crucial function in the development, differentiation and regulation of immune cells. Therefore, dysregulation of cytokine production or action has a central role in the development of autoimmune diseases. Some cytokines, such as TNF and interferon-gamma are potent promoters of immune and inflammatory responses. However, some cytokines have also immunosuppressive functions. The balance between these opposing functions of cytokines has to be tightly regulated by the immune system because excessive production or inadequate inhibition can be life threatening [14].

### 3.1.3 How the immune system communicates with the CNS

Cytokines released in the course of an immune response have to reach the CNS in order to exert their effect on behaviour. Cytokines are water-soluble molecules of relative large molecular weight, and therefore they are not expected to cross the blood brain barrier directly. Different mechanism exist by which peripheral cytokines may gain access to the brain: a) they may enter directly into the brain crossing the specialized endothelial cells composing the blood brain barrier (BBB) by receptor-mediated transport; b) they may reach the brain through areas devoid of a BBB, as in the choroid plexus and the circumventricular organs, comprising the organum vasculosum of the lamina terminalis and the area postrema; c) or they can directly stimulate nerve afferent in the periphery, such as the vagus nerve [12, 15]. Peripheral cytokines can also induce production of brain cytokine synthesis at the endothelial cells, which express receptors for cytokines. Cytokines expressed by these cells may serve as a signal for neurons, astrocytes and microglia cells, which express the cytokine receptors and can selves produce cytokine [16-18].

### 3.1.3 Models of sickness behaviour syndrome

SBS can be induced by several activators of the immune system.

So far, administration of lipopolysaccharide (LPS), mimicking bacterial infection, is the most commonly used model for sickness behaviour. LPS is a component of the cell wall of Gram-negative bacteria which is recognized by TLR4, mainly expressed on monocytes and macrophages. Activation of TLR4 by LPS results in release of the cytokines IL-1, TNF and IL-6 in the periphery as well as in the brain [19-20]. Symptoms of sickness behaviour, such as fever, reduced locomotor activity and weight loss are detected as early as two hours after LPS injection [21]. This effect is clearly dependent on the dose of LPS.

Polyinosinic:polycytidylic acid (Poly I:C) is a synthetic double-stranded RNA and is a ligand for TLR3. Treatment of mice with this compound mimics the acute phase of viral infection, leading to a rapid decrease in open field and burrowing activity, as well as reduction in body weight and core body temperature. These effects are accompanied by increased levels of cytokine expression in the CNS [22].

In the present study we established a new model to study SBS using monoclonal anti-CD40 antibodies. CD40 is a glycoprotein expressed on the surface of B cells, monocytes, macrophages, dendritic cells and microglia, as well as on non-hematopoietic cells such as endothelial cells, fibroblasts, and epithelial cells [23]. CD40 is a member of the tumour

necrosis factor receptor (TNFR) superfamily that also includes TNFR1, TNFR2 and FAS (CD95). Its ligand CD40L (CD154) is a type II transmembrane protein of the TNF gene superfamily (which includes TNFα, lymphotoxin-α and lymphotoxin-β, FasL) mainly produced by activated $CD4^+$ T-cells. Binding of CD40L to its receptor CD40 results in activation of different signalling pathways including the canonical and non-canonical NF-κB-signalling pathway, the mitogen-activated protein kinases (MAPKs), the phosphoinositide 3-kinase (PI3K), which ultimately lead to the induction of cytokines, i.e. TNFα, IL-1β, IL-6 and IL-12, and chemokine expression [24].

The CD40-CD40L interaction on $CD4^+$T-cells is crucial for their priming, expansion and maturation as effector cells. Excessive signalling through CD40-CD40L interaction is associated with numerous autoimmune diseases, including multiple sclerosis, rheumatoid arthritis, and systemic lupus erythematosus [25]. On the other hand, antibodies to CD40 ligand prevented the development of autoimmune diseases in experimental animal models [26]. CD40 mAb antibodies bind to CD40 mimicking the exaggerated CD40 activation observed in autoimmune diseases, therefore, representing a suitable model to investigate sickness behaviour associated with autoimmunity.

### 3.1.4 Tumour necrosis factor

At the end of 19th century William Coley was the first to describe necrosis of tumours induced by bacterial toxins, later on named Coley toxins. In 1975, Carswell and colleagues, while studying hemorrhagic necrosis of tumours produced by endotoxin, found that the serum of *bacillus Calmette-Guérin* (BCG)-infected mice treated with endotoxin contained a substance which mimics the necrotic action of endotoxin itself. They named this molecule, released by macrophages in response to endotoxin, tumour necrosis factor, TNF.

In 1987, TNF was found to be the most relevant cytokine in the development of cachexia, the progressive weight loss and depletion of muscle mass associated with chronic and inflammatory disorders. In this experiment, mice were injected with tumour cells expressing TNF. These mice died quicker and showed dramatic weight loss compared to control mice, indicating that TNF is responsible for the induction of cachexia [27]. In the mean while, TNF has emerged as an important pleiotropic cytokine being one of the mediators of acute phase reactions, endotoxic shock, cachexia in chronic diseases, and cell-mediated host defence against bacteria and viruses, parasites and tumour cells [28].

TNF belongs to the TNF superfamily of ligands, comprising nearly 20 homologues including lymphotoxin-α, lymphotoxin-β, CD40 ligand, and Fas ligand. TNF is synthesized as a monomeric transmembrane protein inserted into the membrane as a homotrimer (mTNF) that can be cleaved by TACE to release a soluble TNF form (sTNF). Both transmembrane and soluble TNF are biologically active. TNF is mainly produced by activated macrophages, but also by lymphocytes, natural killer cells, endothelial cells, microglia, astrocytes and neurons. TNF is a homotrimer of 157 amino acid subunits and signals through two receptors, TNFR1 (p55TNFR) and TNFR2 (p75TNFR), both of which also exist as soluble active forms [29]. The two receptors differ in their expression profiles and ligand affinity. Because their intracellular sequences are largely unrelated, they activate different downstream signalling pathways.

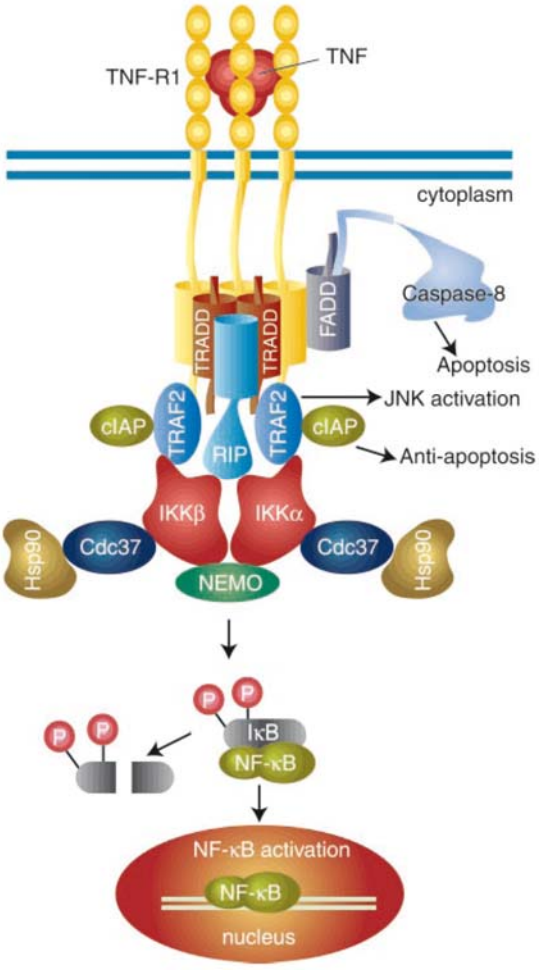


Figure 1. TNFR1 signalling pathway [29].

TNFR1 is expressed in most cell types and preferentially activated by sTNF, although it binds mTNF as well. TNFR1 contains a cytoplasmic death domain (DD) which mediates binding of

TNF receptor associated death domain (TRADD). After binding of TRADD the adaptor proteins receptor interacting protein (RIP) and TNF receptor associated factor 2 (TRAF2) are recruited. This complex leads to activation of the transcription factor NF-κB inducing cellular proliferation, cytokine production and survival signalling [30], and to recruitment of inhibitor of apoptosis protein 1 and 2 (cIAP 1, 2), resulting in activation of ERK, JNK, p38 MAP kinase, and ceramide/sphingomyelinase pathways. The outcome of a TNF signal is determined by the kinetics of JNK activation. Acute and short TNF-induced JNK activation is cytoprotective and is mediated by phosphorylated TAK1, whereas prolonged JNK activation leads to caspase-mediated apoptosis, where JNK is phosphorylated by ASK1 [31]. Furthermore, the TNFR1/TNF complex can be internalized as TNF receptosome, and the death inducing signalling complex (DISC) is formed upon association with FADD and Caspase 8, resulting in TNF-induced apoptosis [32].

Expression of TNFR2 is highly regulated and restricted to cells of the immune system, endothelial cells and some neuronal populations. TNFR2 can be fully activated only by mTNF. Upon TNF binding the adaptor proteins TRAF1 and TRAF2 are recruited to the receptor, leading to activation of cIAPs and NF-κB pathways, promoting survival and inflammation.

TNF is involved in a number of biological activities, ranging from cell growth and death, development and oncogenesis, to immune, inflammatory and stress responses. Implication of TNF in the pathogenesis of a variety of human diseases has been reported, including sepsis, rheumatoid, multiple sclerosis, and inflammatory bowel diseases [29].

Numerous diseases accompanied by severe fatigue, such as plaque psoriasis and rheumatoid arthritis, are associated with elevated levels of TNF in the blood. Etanercept, a soluble TNF receptor that competitively inhibits the interaction of TNF with its receptors, has been successfully applied in the clinic alleviating symptoms of fatigue in patient affected by these diseases [33-34]. So far, only few studies addressing the role of TNF in mediating fatigue have been performed *in vivo*. In an LPS-induced SBS model, blocking of TNF action in the brain by intracerebroventricular injection of soluble TNF receptor reduced the depressive effect of intraperitoneal peripheral injection of LPS. Though, this effect was only seen in IL-1R1 knockout animals and not in wild type, suggesting that TNF might take over the function of IL-1, when this is deficient [35]. Another experiment performed in rat has shown that, plasma collected from animals injected with IL-1β into the hypothalamus is able to decrease locomotor activity when injected into naïve rats. However, the spontaneous motor activity following IL-1β challenge into brain was restored by treatment with Etanercept. This suggests

that behavioural changes induced by IL-1 mediated effects in the CNS may result from peripheral TNF expression [36].

### 3.1.4 Interleukin-1

The IL-1 superfamily is a group of cytokines encompassing IL-1α, IL-1β, IL-1 receptor antagonist (IL-1Ra), IL-18, and IL-33. The primary source of IL-1α and IL-1β are blood monocytes, macrophages and dendritic cells, whereas fibroblasts and endothelial cells normally do not produce these cytokines. They are synthesized as immature proteins which are subsequently cleaved and converted into the mature form. Pro-IL-1α is biologically active and may be cleaved by calpain to be released as mature IL-1α.

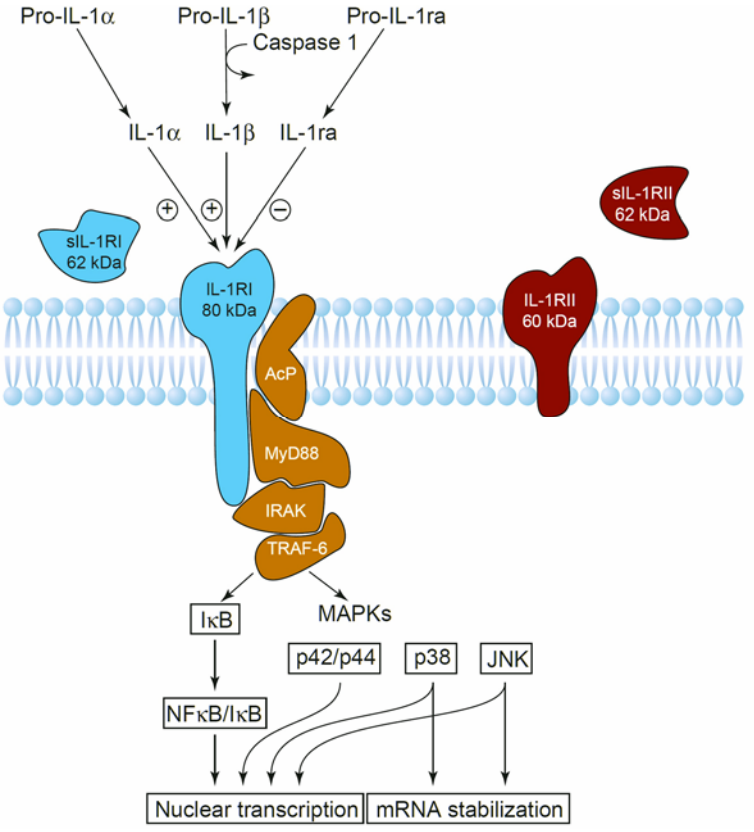


Figure 2. IL-1 signaling pathway [37].

The majority of IL-1α remains intracellular. Pro-IL-1β is biologically inactive and is converted into the mature and active form by Caspase-1, which activity is controlled by a multiprotein complex known as the 'inflammasome'. IL-1β binds to two different receptors, IL-1R1 and IL-1RII. IL-1RII is a negative regulator of the IL-1 system and functions as a decoy receptor, which binds IL-1 with high affinity without transducing any signal. After IL-

1binds to IL-1RI the IL-1 receptor accessory protein (IL-1R-AcP) is recruited to form a complex with IL-1/ IL-1RI, and the cytoplasmic domain of both receptors leads to cell activation. This activated complex recruits different intracellular adapter proteins, including MyD88, IRAK, and TRAF6, activating multiple signalling pathways, including NF-κB, JNK and p38 MAPK [38-39].

The IL-1Ra is structurally similar to IL-1. It binds tightly to the IL-1R1 thereby blocking access of IL-1 to the receptor [38]. Excessive production of IL-1, causing an imbalance between IL-1 and IL1-Ra, is implicated in many autoinflammatory diseases. This pathological increase of IL-1 secretion is usually, but not always, due to dysfunctional activation of caspase-1 by the inflammasome. Anakinra, a recombinant human IL-1Ra, has been approved for the treatment of many IL-1 associated diseases, including rheumatoid arthritis, Still's syndrome and type 2 diabetes [40]. IL-1 is a potent activator of host defence in responses to infection, injury and inflammation mediating fever, slow-wave sleep, sickness behaviour [37], and modulating the neuroendocrine system, especially the hypothalamic pituitary-adrenal axis [41].

Mice deficient in IL-1R1 are fully responsive to sickness-inducing effects of LPS treatment, manifesting increased immobility, and decreased body weight and food intake. Administration of soluble TNF receptor ameliorates these symptoms, though only in IL-1R1 deficient animals and not in wild type controls. Injection of TNF in IL-1R1 knockout animals still induced sickness symptoms. This study by Bluthé et al [35] indicates that both IL-1 and TNF can actually mediate LPS-induced sickness symptoms. It has been shown that IL-1 and TNF share numerous biological activities, indeed. For instance, both have proinflammatory activities, can induce shock-like symptoms, activate the hypothalamic-pituitary adrenal axis, induce fever and hypersomnia and suppress food intake. Moreover, IL-1 and TNF often have additive or synergistic effects [42], they can induce expression of each other and also regulate expression of their own receptors [12].

### 3.1.5 Interleukin-6

Interleukin-6 (IL-6) is produced by various cell types, such as monocytes, B and T cells, fibroblasts, endothelial cells and astrocytes. IL-6 binds to IL-6 receptor (IL-6R) to form the IL-6/IL-6R complex that binds the membrane bound signal transducing protein gp130. The IL-6R also exists in a soluble form which binds IL-6 and forms a complex with gp130 [43]. Engagement of gp130 leads to activation of the receptor-associated kinases JAK1, JAK2, and

Tyk2, and phosphorylation of tyrosine residues in the cytoplasmic tail of gp130, that control the activity of the transcription factors STAT1/STAT3 and the phosphatase SHP2 cascade.
IL-6 is a pleiotropic cytokine that regulates antigen-specific immune responses and inflammatory reactions. It plays a key role in inflammation, being the major inducer of C-reactive protein, fibrinogen and serum amyloid A protein. A variety of studies have demonstrated that overproduction of IL-6 contributes to the pathogenesis of various autoimmune and inflammatory diseases, such as inflammatory bowel diseases, rheumatoid arthritis, osteoporosis and psoriasis [44].

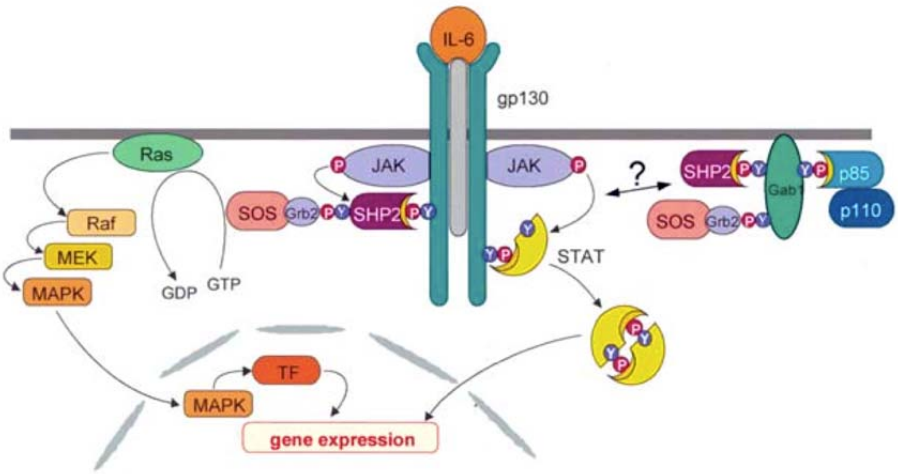


Figure 3. IL-6 signalling pathway [45].

The function of IL-6 in mediating sickness behaviour has been investigated in mice lacking the cytokine. In this study, IL-6 deficient mice were found to be less affected by systemic and central injection of LPS compared to control animals, suggesting a role for IL-6 in the behavioural response to LPS [21].

## 3.2 Circadian rhythms

### 3.2.1 History and meaning of the circadian clock

Until mid of the 19$^{th}$ century it was believed that daily rhythms in behaviour, such as sleep and wakefulness, merely reflects responses to the alteration of light and darkness in the environment. This interpretation was first questioned in 1729 by the French geophysicist Jean

Jacques Ortous de Mairan. Intrigued by the daily opening and closing of the leaves of a *Mimosa* plant, he tested if this biological "behaviour" was simply a response to the sun. To do so, he confined a plant to the dark. The daily rhythmic motions of the leaves persisted even in constant darkness [46]. The existence of an endogenous clock became widely accepted only in the 1950's when Curties Richter observed that the periodicity in the activity of mice was conserved in constant darkness [47]. By now, it is known that daily rhythms in many physiological functions are present in a variety of organisms, from cyanobacteria to *Neurospora* and plants, from *Drosophila* to birds and mammals. The circadian system permits the organism to anticipate periodic events in the environment and to initiate slow processes before they are required. Nearly all aspects of mammalian physiology follow a 24-h cycle. The most striking result of such an organization is the alternation of sleep and wakefulness, but many other functions such as core body temperature, hormone secretion, blood pressure and immune responses, lipid and carbohydrate metabolism, and even cell-cycle progression are organized in such a temporal way [48]. These self-sustained oscillations with periods close to 24 hours are called circadian rhythms.

### 3.2.2 Anatomical basis of circadian rhythms in mammals

Circadian rhythms are generated by autonomously active cellular clocks residing in most tissues of the body, which, in mammals, are coordinated by a master pacemaker located in the suprachiasmatic nuclei (SCN) of the anterior hypothalamus. The SCN is composed of two paired nuclei of approximately ten thousand neurons each, containing autonomous cellular oscillators [49]. In the absence of external time cues, these oscillators generate cycle slightly shorter than 24 hours in mice and slightly longer in humans, and therefore its phase must be daily readjusted. This task is accomplished by the retinal ganglion cells that collect light information via melanopsin photoreceptors and project to the core region of the SCN via the retinohypothalamic tract [50]. The major neurotransmitters involved in resetting the SCN from the retinohypothalamic tract are glutamate and PACAP [51]. Neurons of the SCN include two subpopulations, the one characterized by the expression of the neurotransmitter vasoactive intestinal polypeptide (VIP), the other by the expression of the neurotransmitter arginine vasopressin (AVP) [52].

The key experiment proofing that the SCN is the central pacemaker was performed by Michael Menaker and colleagues [53-54]. They discovered different groups of golden hamsters with different periods in their locomotor activity. Lesion of their SCN caused

arrhythmic behaviour. However, once transplanted with an SCN derived from a hamster with a different endogenous period, the rhythm of the recipient animal was restored. Strikingly, the period was the one of the donor, meaning that the SCN is indeed the organ that imposes the rhythm. Furthermore, when the donor SCN was transplanted in SCN-lesioned hosts encapsulated in a semiporous membrane, preventing in this way the formation of neuronal connections, the circadian rhythm of locomotor activity was still restored [55]. This implies a diffusible factor that is able to deliver signal s from the SCN to the periphery. Recently, different molecules, such as transforming growth factor-alpha (TGF-α) [56], prokineticin-2 (PK2) [57] and cardiotrophin-like cytokine (CLC) [58], have been found to be secreted in a circadian way and are able to inhibit spontaneous locomotor activity. The rhythms of melatonin and corticosteroid secretion have been shown to need synaptic connections [59-60]. Projections from the SCN to nearby hypothalamic structures and brainstem then coordinate behaviour and physiology. Neurons within the dorsal subparaventricular zone (SPZ) of the hypothalamus are necessary for organizing circadian rhythms of body temperature, whereas neurons in the ventral SPZ are needed for rhythms of sleep and waking. The ventral SPZ in turn relays to the dorsomedial nucleus, which generates circadian rhythms of sleep and waking, locomotor activity, feeding and corticosteroid production [61].

Feeding time is the major Zeitgeber that entrains the phase of peripheral oscillators. Feeding mice exclusively during the day gradually inverses the phase of circadian gene expression in peripheral organs such as liver, pancreas, skeletal muscle and kidney, while the phase of the SCN remained unaffected [62]. However, as soon as food is available ad libitium, the peripheral clocks are quickly resynchronized with the SCN. Because this resetting is much faster than desynchronization when shifting feeding from night to day time, it is thought that the SCN might also use direct cues to synchronize peripheral clocks. Glucocorticoid signalling has been proposed as messenger molecule in this process [63].

### 3.2.2 The molecular organization of the mammalian clock

At the molecular level, the mammalian clock consists of interacting positive and negative transcriptional feedback loops that drive rhythmic transcription and translation of key clock components (reviewed in [64]). In this model, heterodimers of CLOCK and BMAL1 proteins bind to E-box regulatory DNA sequences to activate the expression of *Per* (*Period 1*, *2*) and *Cry* (*Cryptochrome 1*, *2*) genes at the beginning of the circadian day. Approximately 12 h later, when PER and CRY levels have reached their highest concentration in the cytoplasm,

they form heterodimers that enter the nucleus and bind to the CLOCK:BMAL1 complex, suppressing E-box driven gene transcription. When the levels of *Per* and *Cry* transcripts decrease, the inhibition of the CLOCK:BMAL1 complex is released and another cycle can begin. *Clock* mRNA and protein are constitutively expressed, whereas *Bmal1* mRNA expression cycles in antiphase to that of *Per* and *Cry*. This delayed expression of *Bmal1* depends on an accessory loop involving the nuclear orphan receptors, REV-ERBα and RORα. Because *Rev-Erbα* and *RORα* bear E-box sequences in their promoters, their expression is also driven by CLOCK and BMAL1 heterodimers and therefore rhythmically in phase with *Per* and *Cry*. REV-ERBα and RORα bind to the ROR element of the *Bmal1* promoter through RORE sequences, respectively inhibiting and activating *Bmal1* transcription [65] (Fig 4).

CLOCK:BMAL1-mediated transactivation is associated with rhythmic changes in histone acetylation, suggesting that chromatin remodelling is an important regulatory mechanism in the circadian clock machinery. The CLOCK protein itself possesses an intrinsic acetyltransferase activity specific for histone and BMAL1. The acetyltransferase activity of CLOCK is counterbalanced by sirtuin 1 (SIRT1), which deacetylases PER2 and BMAL1 [66]. Besides direct transcriptional control, clock proteins are regulated at the posttranslational level through various modificatons, such as phosphorylation [67], sumoylation [68], histone acetylation [69] and methylation [70], which regulate the localization, degradation and activity of the core clock proteins, contributing to the generation of a 24 hours period.

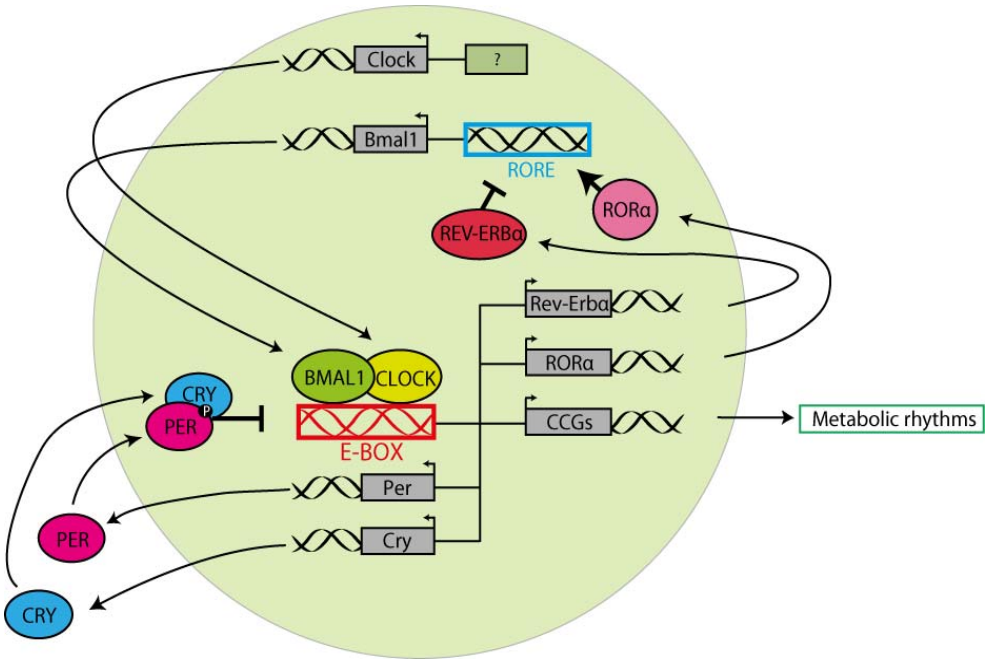


Figure 4. The molecular clock, adapted from [65].

### 3.2.3 The output of the clock

The circadian oscillator regulates behavioural, physiological, and metabolic rhythms by activating output pathways at the appropriate time of the day. Microarray studies revealed that 3-10% of all expressed transcripts are under circadian regulation [71]. Rhythmic genes are expressed in a tissue-specific manner, with only a minor overlap between tissues, reflecting the tissue specificity of different rhythmic outputs [72]. These transcripts include key and rate limiting enzymes involved in various aspects of physiology and metabolism, such as protein synthesis and turnover, energy production and nutrient metabolism. In the liver, for example, rate-limiting steps of glycolysis, fatty-acid metabolism, cholesterol biosynthesis, and xenobiotics metabolism are under tight circadian control.

These rhythmically expressed downstream genes are called clock-controlled genes (CCGs) and their expression may be directly or indirectly depend on core clock components. Directly controlled genes harbor E-boxes or ROR elements in their promoter, and therefore depend on CLOCK:BMAL1 complexes and REV-ERBα respectively for activation. Among the CCGs directly controlled by CLOCK are the members of the PAR-bZip (proline and acidic amino acid-rich basic leucine zipper) transcription factor family: *Dbp* (*D-site albumin promoter binding protein*), *Tef* (*Thyrotroph embryonic factor*) and *Hlf* (*Hepatic leukemia factor*). These proteins activate transcription of downstream genes bearing the consensus sequence RTTAYGTAAY [73]. All three PAR bZIP genes are cyclically expressed in the SCN [74]. DBP and TEF accumulate in most cell types, whereas expression of HLF is restricted to liver, kidney and brain. When all three genes are inactivated, most of the mice die because of spontaneous epileptic seizures shortly after birth. This might be explained by reduction of *Pyridoxal Kinase* (*Pdxk*) expression observed in these mice. PDXK converts vitamin B6 derivatives into pyridoxal phosphate (PLP), an essential co-enzyme involved in the metabolism of different neurotransmitters, like serotonin and dopamine. These mice have indeed lower amounts of these neurotransmitters, which may be the cause for the lethal epileptic seizures. Furthermore, triple knockout mice for DBP, HLF and TEF, also show signs of early aging, and less than 20% of the animals survive longer than one year. DBP, HLF and TEF have been found to control transcription of enzymes regulating defence against xenobiotics and oxidative stress, explaining the strong phenotype observed in triple knockout animals.

Of the three PAR transcription factors, DBP is the one with the highest abundance and the largest circadian oscillation. Studies in *Dbp* knockout mice evidenced a role for DBP in

controlling circadian behaviour, although its precise role in the central pacemaker is still unknown. Mutant mice still exhibit a circadian behaviour, but have a 30 minutes shorter period and are 64% less active than wild type animals [74].
Furthermore, the DBP deficiency affects the distribution of sleep episodes during the day with more episodes during the active period. These mice also had lower level of slow wave sleep delta power, which reflect a homeostatic process underlying the regulation of SWS propensity [75].

### 3.2.4 The clock and its impact on physiology

As mentioned above, circadian rhythms affect a variety of physiological processes and disruption of normal circadian biology has been associated with a series of pathologies, including cancer, sleep and metabolic diseases.
Since the cycle of cell division is under circadian control [76], it is not surprising that cancer has been suggested to be a circadian-related disorder. Mutations in some clock genes are associated with increased cancer risk. A polymorphism in *Per3* has been linked to early onset of breast cancer in women, and abnormalities in *Per1*, *Per2* and *Per3* expression are observed in tumours [77]. Furthermore, tumours grow faster in mice that have their SCN ablated [78], or mice that are exposed to irregular night-dark cycles [79], and shift work or jetlag may contribute to a higher risk of cancer and increased mortality in humans [80].
Some of the circadian master genes have been shown to influence sleeping behaviour. A point mutation in human *Per2* has been found in subjects affected by advanced sleep phase syndrome (FASP), a disorder characterized by very early sleep onset and offset [81-83]. Circadian rhythm abnormalities have also been described in unipolar depressive and bipolar disorders [84]. Animals with mutations in clock genes have been found to have metabolic disorders. For instance, homozygous *Clock* mutant mice have altered feeding rhythms and develop metabolic syndrome with obesity, hyperlipidemia, hyperglycemia, and hyperinsulinemia and diabetes [85]. In addition to disruption of circadian function, *Bmal1* deficiency results in reduced body weight, early aging accompanied by sarcopenia, osteoporosis, reduced lifespan and peripheral blood composition [86]. Moreover, conditional ablation of pancreatic clock in pancreas-specific *Bmal1* mutant mice leads to increased glucose tolerance and increased insulin resistance, resulting in hyperglycaemia [87]. The importance of the circadian system in regulating multiple physiological and metabolic

processes is evidenced by the phenotype observed in mutant mice. These are summarized in table 1.

Table 1, adapted from [88].

| Gene | Circadian phenotype | Associated physiological abnormality |
|---|---|---|
| Bmal1/Mop3 null mutant | Loss of circadian activity rhythm in DD | Infertility, decreased adult body weight, increased tendon calcification, abnormal gluconeogenesis and lipogenesis, premature aging syndrome, increased sleep fragmentation |
| Clock $^{\Delta 19}$ | 4 h longer period followed by loss of circadian activity in DD | Hyperphagic and obese, abnormal gluconeogenesis, mania phenotype, decreased duration of sleep time |
| Clock null mutant | 0.5 h shorter period | N.D. |
| Per1 null mutant | 0-0.5 h shorter period | Lack of sensitization to cocaine |
| Per2, Per2$^{tm1Brd}$ | 1.5 h shorter period and tendency for loss of circadian rhythm | Increased tumour development following genotoxic stress, improper alcohol intake, early onset of sleep |
| Per1 and Per2 double null mutant | Complete loss of circadian activity rhythm in DD | N.D. |
| Per3 null mutant | 0-0.5 h shorter period | N.D. |
| Cry1 null mutant | 1 h shorter period | N.D. |
| Cry2 null mutant | 1h shorter period | N.D. |
| Cry1 and Cry2 double mutant | Complete loss of circadian activity rhythm in DD | Delayed hepatocyte regeneration, increased resistant to chemotherapeutic agent's toxicity, increased NREM sleep drive |
| *Rev-Erbα* null mutant | 0.5 h shorter period, altered photic entrainment | N.D. |

### 3.2.5 The circadian clock and the immune system

A number of lines of evidence suggest that cytokines and other immune mediators can influence circadian timekeeping processes, and likewise that the immune system may be under the circadian control.

The circadian clock has been characterized in peritoneal macrophages, and the expression of several clock genes such as *Bmal1*, *Rev-Erbα*, *Per1*, *Per2* and *Dbp* exhibited diurnal variation. Furthermore, the expression of the inflammatory factor MCP-1 (monocyte chemoattractant protein-1) as well as the phagocytic activity of isolated macropahges showed a robust circadian pattern [89]. Fully competent circadian clocks also exist in splenocytes and lymph nodes. Secretion of TNF and IL-6 by spleen macrophages challenged with LPS also displayed a significant circadian oscillation. In addition, as revealed by microarray analysis, approximately 8% of the genes expressed in macrophages are under circadian regulation. Among these were canonical clock genes like *Bmal1*, *Per1*, *Per2*, *Per3*, *Rev-Erbα*, *Cry1*, *Cry2* as well as *Dbp* [90]. Different functions of natural killer (NK) cells, such as expression of the cytolytic factors Granzyme B and Perforin, as well as the cytokines TNF and IFNγ have been reported to follow a circadian pattern [91].

Recently, Cavadini et al [92] have shown that TNF directly regulates the expression of E-box driven clock genes, such as *Per1*, *2*, *3* as well as *Dbp*, *Hlf* and *Tef* in cultured fibroblasts. This effect on clock gene expression was also found in liver of TNF treated mice and was accompanied by an increase in rest episodes.

The effect of intravenous injection of LPS on clock genes has been investigated in rats. This study showed that LPS suppresses the expression of *Per* and *Dbp* in the SCN, and downregulates the expression of *Per1*, *Per2*, *Dbp* and *PPARα* in the liver of treated animals [93]. Taken together, these findings lead to the question whether and to what extent the behavioural effects of centrally acting cytokines and other immune mediators are mediated via the circadian clock.

## 3.3 Aim of the project

The role of the immune system in clearance of pathogens and tissue destruction in infectious and autoimmune diseases has been mostly clarified. Though, the molecular mechanisms underlying the physiological, metabolic, and behavioural changes evoked by the activated immune system have been poorly investigated so far.

Here, we aimed at establishing a mouse model for studying SBS associated with chronic T-cell dependent inflammation. Agonistic anti-CD40 antibodies were used to mimicking the action of T-cell derived CD40 ligand. These antibodies are known to induce the full spectrum of sickness behaviour symptoms and to activate the peripheral immune system inducing the synthesis TNF, IL-1, and IL-6. We especially focus on locomotor activity behaviour and weight loss as read out for SBS.

To address the role of TNF, IL-1, and IL-6 in the induction of SBS anti-CD40 antibodies were applied to mice deficient for either the cytokine or the cytokine receptor. Moreover, the impact of CD40-mediated immune activation on the molecular clock was assessed.

# 4. RESULTS

## 4.1 CD40 induced Sickness Behaviour depends on TNF signalling and associates with altered clock gene expression

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**SUMMARY**

Infectious and autoimmune diseases are associated with fever, fatigue, reduced appetite,and depression. These symptoms are collectively referred to as sickness behaviour syndrome (SBS). SBS is particularly debilitating in many autoimmune diseases including, multiple sclerosis and rheumatoid arthritis. It is a common belief that these symptoms are mainly caused by the action of cytokines TNF-α, IL-1β and IL-6. However, it is still unclear which cytokine is essential for the development of SBS. Moreover, the mechanisms leading to the SBS have been poorly investigated so far.

Here we present a model for investigating SBS associated with autoimmune diseases using anti-CD40 antibodies. In this model SBS evoked by CD40 activation is mediated by TNF:TNFR1 interaction and does not depend on IL-1R1 or IL-6. The intensity of the CD40 evoked SBS correlates with suppression of E-box controlled clock genes including *Dbp*, *Bmal1* and *Rev-Erbα*. Thus, our results suggest that SBS development in autoimmune diseases is dependent on CD40-mediated TNF expression and indicate a role for dysregulation of the circadian clock.

## INTRODUCTION

Infectious and autoimmune diseases are associated with fever, fatigue, reduced appetite and depression. These symptoms are referred to as sickness behaviour syndrome (SBS). SBS is considered an adaptive strategy to better cope with infections, to facilitate recovery, and to decrease the risk of disease transmission among individuals. In the course of autoimmune diseases, including multiple sclerosis, lupus erythematosus (SLE), and rheumatoid arthritis (RA), SBS is rather maladaptive, and often represents the most debilitating effect compromising the quality of life of affected individuals. For instance, incidence of fatigue has been reported in 91% of SLE patients [94], and severe fatigue, perceived as frustrating or exhausting, is experienced in 42% of RA patients [7]. Many lines of evidence indicate that SBS is induced and maintained by cytokines such as tumor necrosis factor alpha (TNF), interleukin (IL-) 1 and -6, which are produced in the course of an immune response [12]. Clinical observations suggest a causal link between TNF and fatigue in autoimmune diseases. Treatment with the soluble TNF receptor p75 improved fatigue in patients with RA [33, 95]. Moreover, in multiple sclerosis, fatigue correlated with TNF expression in blood leukocytes [6]. However, it is presently unclear which cytokine-cytokine receptor system is responsible for SBS.

Since no animal model exists to investigate the pathogenesis of SBS in autoimmune diseases, we established a new mouse model using agonistic antibodies to CD40. CD40 receptor is expressed on B lymphocytes and antigen presenting cells (APC), namely dendritic cells and macrophages. Its binding to CD40 ligand (CD40L, CD154), secreted by activated $CD4^+$ T lymphocytes, stimulates APC to produce cytokines such as TNF, IL-1β, and IL-6 [23-24, 96]. Antibodies against CD40L prevent $T_H1$-mediated autoimmune diseases including SLE and RA Durie [26, 97-98]. Antibodies to CD40 or soluble CD40L have therapeutic potential in the treatment of lymphoproliferative malignancies and solid tumours. However, their applicability is limited due to the occurrence of SBS with fatigue, anorexia and headache [99].

The molecular mechanisms leading to the physiological, metabolic, and behavioural changes observed in SBS are presently unclear. Circadian dysfunction is thought to contribute to the incidence of a wide range of diseases, including sleep disorders and depression. Circadian rhythms are maintained by basic helix-loop-helix (bHLH)-PAS transcription factors CLOCK and BMAL1, which bind as a dimeric complex selectively to E-boxes ($CACGT^G/_T$) in the promoters of the *Per* (*Per 1-3*) and *Cry* (*Cry 1-2*) genes and of clock-controlled genes (CCGs) such as the PAR bZIP transcription factors *Dbp*, *Tef* and *Hlf* [64, 100]. PER and CRY proteins translocate to the nucleus and repress the activity of CLOCK and BMAL1, thereby

inhibiting their own transcription. The transcription of *Rev-Erbα* is positively regulated by CLOCK:BMAL1 and negatively by PER2. REV-ERBα binds to the ROR elements (ROREs) in the *Bmal1* promoter driving the rhythmic transcription of the latter [101].

Recently, we found that TNF impairs the expression of E-box driven clock genes, including the PAR bZip transcription factors *Dbp*, *Tef* and *Hlf*, as well as *Per*, *Cry* and *Rev-Erbα*. The effect of TNF is seen in murine fibroblasts in-vitro and upon subcutaneous infusion of TNF in also in livers of treated mice [92]. Inducers of TNF, namely LPS, have also been shown to suppress the expression of clock genes [93]. These data indicate a possible crosstalk between the circadian clock and the cytokine network.

The aim of our study was to investigate which cytokine is essential for the development of SBS and to assess the impact of CD40-mediated immune activation on the expression of clock genes.

## RESULTS

### CD40 ligation induces TNFR1-dependent changes in behaviour

Wildtype (wt, C57BL/6J) mice were treated with agonistic CD40 monoclonal antibodies (CD40 mAb) intraperitoneally and their locomotor activity was monitored using running wheels and infrared activity detection devices. Significant reduction in locomotor activity was observed in CD40 mAb treated wt mice compared to mice receiving isotype control antibodies. Marked reductions were seen one day post injection between ZT17 - 21 (Fig. 1 and Supplementary Fig. 1). Impaired locomotor activity was evident in the 12-h dark period mainly due to an increase in rest episodes - indicators of sleep induction - lasting more than 5 minutes (Supplementary Fig. 3). During the light period, when mice usually rest, no changes in locomotor activity or rest were observed, indicating that the daily distribution of activity remained within the normal pattern. To assess the contribution of TNF, IL-1β and IL-6, the main actors in the development of SBS, CD40 mAb were injected into mice with a deletion of the *Tnfr1*, the *Il1r1*, and the *Il6* gene. As seen in wt mice, IL-$6^{-/-}$ mice responded with a significant impairment of locomotor activity and an increase in rest episodes longer than 5 min. (Fig. 1, Supplementary Fig. 1, 2 and 3). Likewise, the deletion of IL-1R1 did not protect mice from CD40 ligation-induced SBS. However, the effect in IL-$1R1^{-/-}$ was less pronounced compared to wt (Fig. 1, Supplementary Fig. 1, 2 and 3). TNFR1 deficient mice treated with CD40 mAb showed almost complete protection from loss of locomotor activity as measured by running wheel (Fig. 1) and infrared activity (Supplementary Fig. 1). Likewise, rest episodes were not increased (Supplementary Fig. 3). Heterozygous TNFR1 mice showed intermediate reduction of activity suggesting a gene dosage effect of TNFR1 (Supplementary Fig. 4). Taken together, TNFR1 seems to be essential in CD40-mediated fatigue as reflected by locomotor activity.

Taking into account all the reports on SBS mediated by LPS and IL-1β, we assessed the effect of CD40 mAb in MyD$88^{-/-}$ mice. MyD88 is an intracellular adaptor molecule, mainly used by TLRs (except TLR3), IL18R and IL-1R to activate the transcription factor NF-κB as well as interferon-responsive factors [102]. Surprisingly, MyD$88^{-/-}$ mice were resistant to CD40 mediated SBS as evidenced by their locomotor activity, and rest episodes (Fig. 2, Supplementary Fig. 2 and 3). Heterozygous MyD$88^{+/-}$ mice were fully susceptible to the respective effect of CD40 mAb (Supplementary Fig. 4). Resistance of MyD$88^{-/-}$ mice is unlikely to depend on IL-1 signalling because, as shown above, IL-$1R1^{-/-}$ mice did develop SBS.

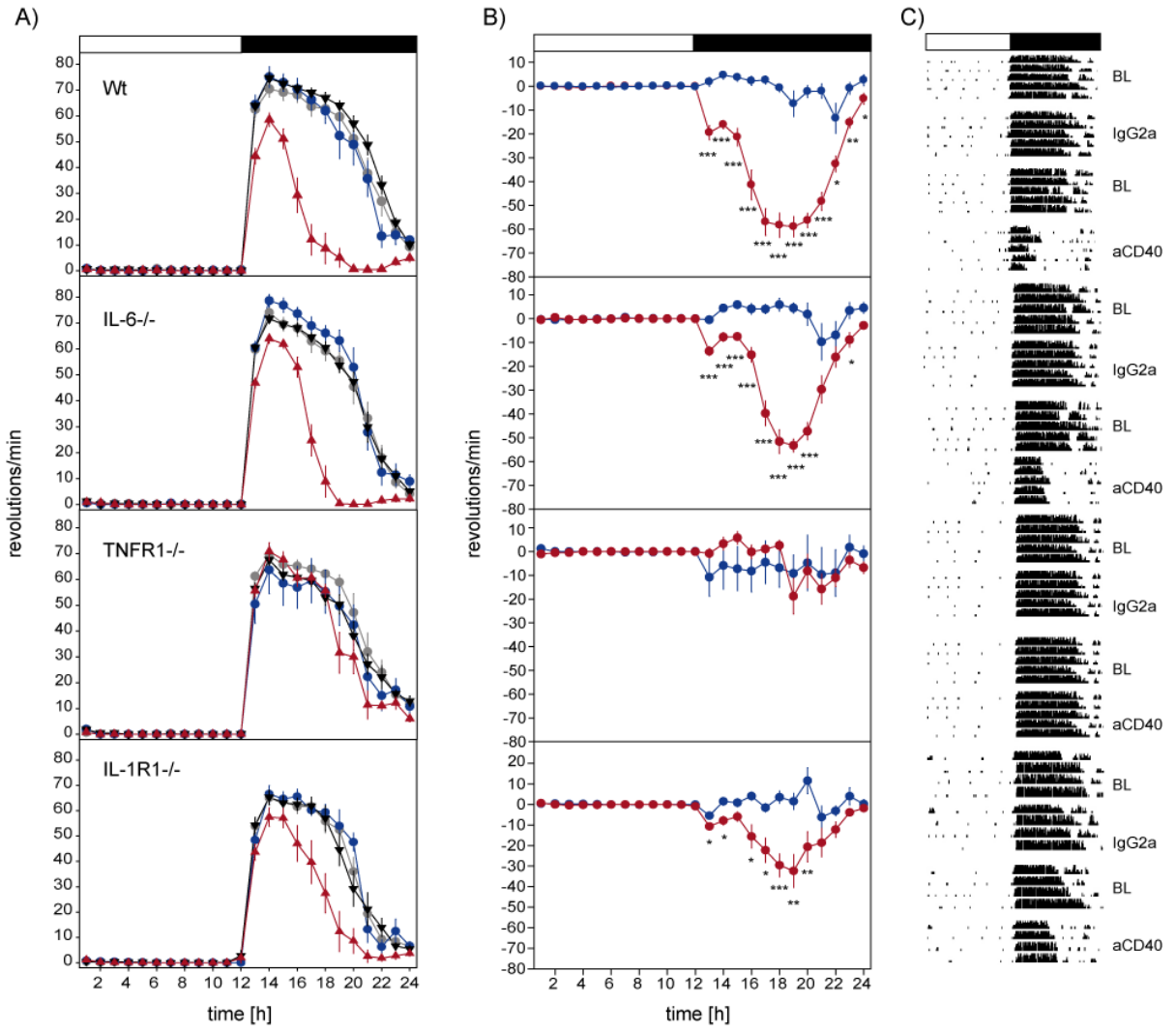


**Figure 1. CD40 ligation does not affect locomotor activity in TNFR1$^{-/-}$ mice.**

A) Running wheel activity for the first day after i.p. injection of 200 µg CD40 mAb (red line) or IgG2a as control (blue line). Baselines are the mean of three days preceding injection of CD40 mAb (black line) or IgG2a control (gray line). Mean of 1-h values ± s.e.m. Note the reduced activities upon CD40 mAb treatment (red lines) in wt, IL-6$^{-/-}$, and IL-1R1$^{-/-}$ are not present in TNFR1$^{-/-}$ mice. B) Difference in running wheel activity after subtracting the baseline. Blue line: IgG2a; Red line: CD40 mAb. The data are shown as means ± s.e.m. Stars indicate 1-h interval which differed significantly between treated and baseline groups: *P< 0.05, **P <0.005, ***P< 0.0005, 2-tailed t-test after significant 2-way ANOVA for 'interval' and 'treatment'. C) 24-h actograms of running wheel activity of five representative mice per group. Each line represents one individual mouse, one day before (baseline, BL) and one day after injection of IgG2a and CD40 mAb. The light-dark period is indicated by the black and white bars at the top of the plot. Data are from wt (IgG2a), n = 10; wt (CD40 mAb), n = 12; IL-6$^{-/-}$ (IgG2a), n = 9; IL6$^{-/-}$ (CD40 mAb), n = 11; TNFRI$^{-/-}$ (IgG2a), n = 10; TNFR1$^{-/-}$ (CD40 mAb), n = 11; IL-1R1$^{-/-}$ (IgG2a), n = 11; IL-1R1$^{-/-}$ (CD40 mAb), n = 11; for A and B.

We speculated that TNF may be involved in the phenotype observed when using MyD88$^{-/-}$ mice because (1) MyD88$^{-/-}$ mice have been shown to be low producers of TNF in response to TLR activation and, (2) we find CD40 ligation-induced SBS to depend on TNFR1. Indeed, treatment of MyD88$^{-/-}$ mice with CD40 resulted in significantly lower TNF concentration in the serum compared to wt mice (Supplementary Fig. 5). To address if low level of TNF production is responsible for resistance to SBS induction, we administered TNF to MyD88$^{-/-}$ mice using osmotic minipumps placed subcutaneously. As observed in wt mice, TNF was also able to restore CD40-mediated SBS in MyD88$^{-/-}$ mice (Fig. 2). Thus, TNF seems to be the crucial factor in the induction of SBS in MyD88$^{-/-}$ mice.

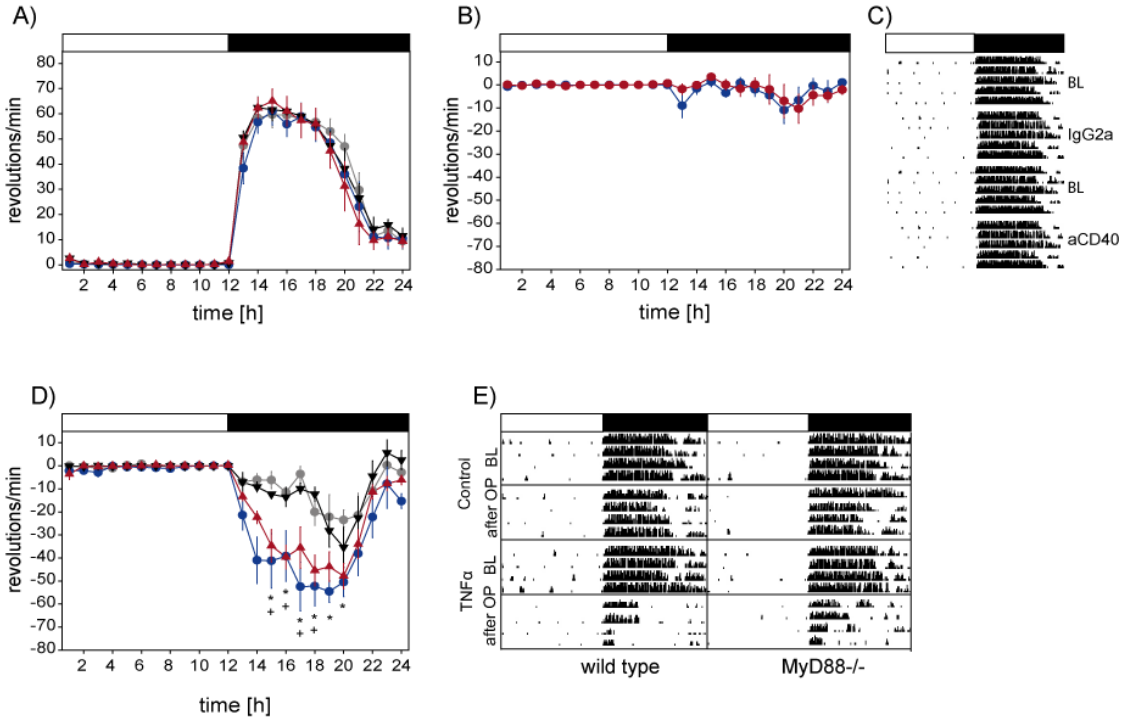


**Figure 2. Sickness behaviour syndrome does not develop in CD40 mAb treated MyD88 -/- mice, but can be induced by TNF.** A) Running wheel revolutions per hour in CD40 mAb treated MyD88$^{-/-}$ mice (red line) and IgG2a control (blue line) and the respective baseline for CD40 mAb (black line) and control IgG2a (gray line). No effects in CD40 treated mice. B) Difference in running wheel activity after subtracting the baseline. Blue lines: IgG2a; red lines: CD40 mAb. The data are means ± s.e.m. C) 24-h actograms of running wheel activity of five representative mice per group. Each line represents one mouse, one day before (baseline, BL) and one day after i.p. injection of IgG2a and CD40 mAb. D) Changes in locomotor activity three days after implantation of the minipump, which releases TNF (1.5 µg/day) or saline as control, compared with the baseline (mean of three days immediately before the implantation). Gray line: wt baseline; Black line: MyD88$^{-/-}$ baseline; Blue line: wt TNF infused; Red line: MyD88$^{-/-}$ TNF infused. Stars (wt) and crosses (MyD88$^{-/-}$) indicate 1-h intervals which differed significantly between TNF-infused and saline groups: *,+$P < 0.05$, 2-tailed t-test after significant 2-way ANOVA for 'interval' and 'treatment'. E) 24-h actograms of running wheel activity of five individual mice before and after minipump implantation. Each line represents one individual mouse, one day before (baseline,

BL) and 3 days after the implantation (OP). The light-dark period is indicated by the black and white bars at the top of the plot. The data are from MyD88$^{-/-}$ (IgG2a), n = 8; MyD88$^{-/-}$ (CD40), n = 10; for A and B. Or from MyD88$^{-/-}$ (Saline), n = 5; MyD88$^{-/-}$ (TNF), n = 5; for D.

In wt mice the effect on locomotor activity was associated with significant weight loss (-11.7% ± 3.5) compared to isotype-treated animals (-1.9% ± 1.7, P < 0.001) at day two post injection (Fig. 3). Whereas mice deficient for IL-6 showed dramatic weight loss (-9.9% ± 1.9, P < 0.001), IL-1R1 deficient mice had much less reduction in body weight (-4.0% ± 2.1, P = 0.003). In TNFR1 deficient mice weight loss was only minimal, but still significant (-2.1% ± 2.3, P = 0.01), and TNFR1 heterozygous mice developed intermediate weight loss (-4.4% ± 2.9, P < 0.005). Interestingly, MyD88$^{-/-}$ mice were completely resistant to CD40- induced weight loss (-2.3% ± 2.1, P = 0.3). Thus, it certainly appears that TNFR1 and IL-1R1 are critically involved in inflammation-induced weight loss triggered by CD40 ligation, while IL-6 has no effect in this respect. In contrast to the loss of body weight, which depended on the knockout mouse line used, CD40 ligation-induced splenomegaly was seen independent of the genotype (Fig 3).

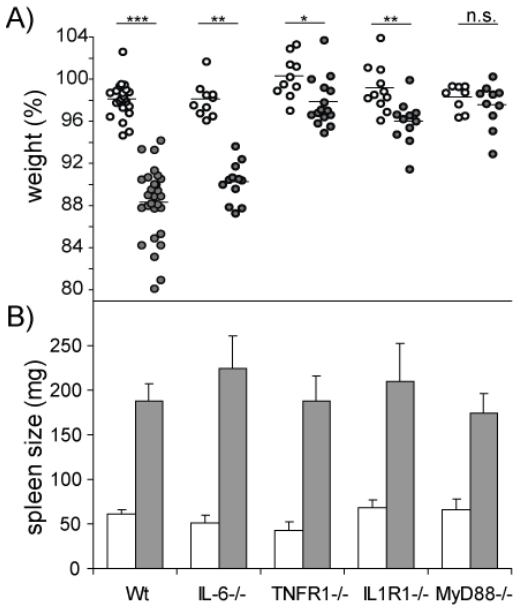


**Figure 3. CD40 induced weight loss, but not the increase of spleen size, differs between genotypes.** A) Body weight measured two days post injection of CD40 mAb (filled dots) or control IgG2a (open dots), is shown as percent of body weight before injection (100%). *P< 0.05, **P< 0.005, ***P< 0.0005, Mann-Whitney test. B) Spleen weight measured two days post injection. White bars: IgG2a; Gray bars: CD40 mAb. The data are means + SD. Data are from wt (IgG2a), n = 10; wt (CD40), n = 12; IL-6$^{-/-}$ (IgG2a), n = 9; IL6$^{-/-}$(CD40), n = 11; TNFRI$^{-/-}$ (IgG2a), n = 10; TNFR1$^{-/-}$ (CD40), n = 11; IL-1R1$^{-/-}$ (IgG2a), n = 11; IL-1R1$^{-/-}$ (CD40), n = 11; MyD88$^{-/-}$ (IgG2a), n = 8; MyD88$^{-/-}$ (CD40), n = 10; for A and B.

**Impaired clock gene expression in CD40 mAb treated mice**

We next asked whether CD40 ligation, besides inducing SBS in a TNFR1-dependent way, might also lead to altered expression of clock genes. We have recently identified TNF to decrease the expression of clock genes in mice [92]. CD40 ligation in livers of wt mice was followed by an increase of *Bmal1* expression with upregulation at ZT5 and ZT9 (Fig. 4). The expression of *Clock* was downregulated at ZT1 and ZT5. *Rev-Erbα*, *Per2* and *Per3* clearly show attenuated expression of their amplitudes. The expression of the clock-output genes *Dbp*, *Hlf*, *Tef* was dramatically suppressed at the time points where the expression of the respective genes showed a peak (Fig. 4). In the kidney, similar changes, namely upregulation of *Bmal1,* and marked down regulation of *Dbp*, *Hlf*, *Tef*, *Rev-Erbα*, and *Per3*, were detected (Supplementary Fig. 6). Thus, the effect of CD40 ligation on clock gene expression is not restricted to the liver.

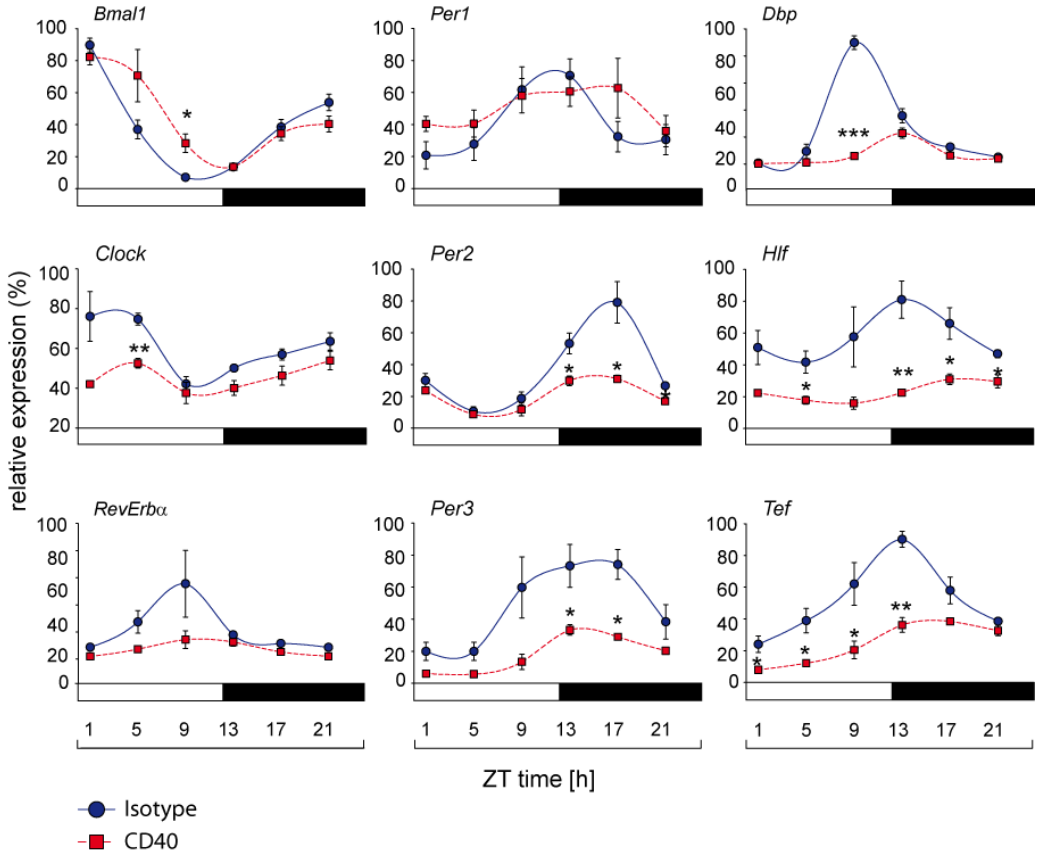


**Figure 4. Circadian gene expression of clock and clock-controlled output genes are severely impaired by CD40 treatment.** Gene expression in mouse liver during a 24 h time period (one day p.i., ZT0 lights on, ZT12 lights off) quantified by RT-PCR. CD40 mAb induce *Bmal1* at ZT5 and ZT9, reduce *Clock* and reduce amplitudes of the E-box dependent clock genes *Rev-Erbα*, *Per2*, *Per3*, and clock-output genes *Dbp*, *Hlf*, and *Tef.* Three mice per time point and treatment group were analyzed. Mean ± s.e.m. Student's t-test. * P< 0.05, ** P< 0.005, *** P< 0.0005.

In further experiments we assessed the effect of CD40 ligation on *Dbp*, *Rev-Erbα* and *Bmal1* at ZT9 two days after injection in the same knockout mice which we had used to assess locomotor activity. IL-$6^{-/-}$ mice showed similar expression pattern as seen in wt mice (Fig. 5). In $TNFR1^{-/-}$ mice downregulation of *Dbp* and *Rev-Erbα* and upregulation of *Bmal1* was less pronounced compared to wt mice. $TNFR1^{+/-}$ heterozygous mice even had a stronger suppression of *Dbp* and *Rev-Erbα* and a stronger induction of *Bmal1* compared to $TNFR1^{-/-}$ mice. As was the case for $TNFR1^{-/-}$ and $TNFR1^{+/-}$, also $MyD88^{-/-}$ and $MyD88^{+/-}$ differed in the response to CD40 ligation, suppression of *Dbp* and *Rev-Erbα* and increase in *Bmal1* being more pronounced in heterozygous mice (Fig. 5).

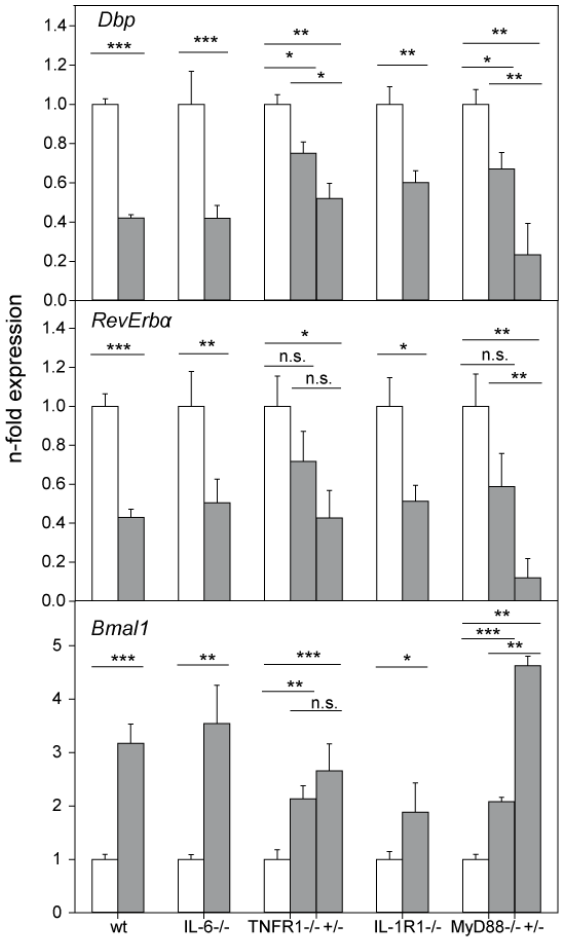


**Figure 5. $MyD88^{-/-}$ and $TNFR1^{-/-}$ mice are less prone to CD40 mAb induced dysregulation of clock gene expression.** Gene expression in liver of mice held in a 12-h light/12-h dark cycle and sacrificed 2 days after i.p. injection of Ig2a (white bars) or CD40 mAb (gray bars) at ZT = 9-10 (ZT = 0, light on, ZT = 12, light off), quantified by RT-PCR. Besides of $TNFR1^{-/-}$ and $Myd88^{-/-}$ mice also their heterozygous littermates were analysed (+/-). Data are means + s.e.m. *P< 0.05, **P< 0.005, ***P< 0.0005, Mann-Whitney test. Data are from wt (IgG2a), n = 10; wt (CD40), n = 12; IL-$6^{-/-}$ (IgG2a), n = 9; $IL6^{-/-}$ (CD40), n = 11; $TNFRI^{-/-}$ (IgG2a), n = 10; $TNFR1^{-/-}$ (CD40), n = 11; $TNFR1^{+/-}$ (CD40), n = 6; IL-$1R1^{-/-}$ (IgG2a), n = 11; IL-$1R1^{-/-}$ (CD40), n = 11; $MyD88^{-/-}$ (IgG2a), n = 8; $MyD88^{-/-}$ (CD40), n = 10; $MyD88^{+/-}$ (CD40), n = 6.

To validate the importance of TNF:TNFR1 interaction in CD40-mediated alterations of expression of clock genes, we investigated the effect of CD40 ligation on DBP, REV-ERBα and BMAL1 at the protein level (Supplementary Fig. 7). Treatment of wt mice, with CD40 mAb was followed by significant downregulation of DBP and REV-ERBα in nuclear extracts of livers at ZT9 day two post injection. The NF-κB subunit p65, which is translocated to the nucleus upon immune activation, is upregulated. BMAL1 and CLOCK were unaffected. In $TNFR1^{-/-}$ mice only REV-ERBα was significantly downregulated, but significance was less than in wt. NF-κB showed a trend to upregulation. By comparing $TNFR1^{-/-}$ with $TNFR1^{+/-}$ mice both treated with CD40 mAb, we detected differences in DBP, REV-ERBα, and BMAL1 expression, which was significantly decreased in heterozygous mice compared to monozygous TNFR1 knockout mice; no differences were seen for p65 and CLOCK. To evaluate the effect of CD40 mAb treatment on chromatin remodelling and the binding status of the transcription factors CLOCK and BMAL1 at the E-box sequences of the *Dbp* and *Rev-Erbα* promoters we established chromatin immunoprecipitation (ChIP) assays. Histone H3 acetylation at Lys9 (AcH3K9) has been reported to be rhythmic in the *Per1* and *Per2*, and the *Dbp* gene [103] being highest at ZT9 when transcriptional activity is at its maximum. In addition, rhythmic CLOCK:BMAL1 binding to the *Dbp* gene has been shown in detail. In CD40-treated wt mice, reduced levels of AcH3K9 were measured at the E-boxes of the *Dbp* and *Rev-Erbα* genes (Fig. 6). CD40-treated wt mice, reduced levels of AcH3K9 were measured at the E-boxes of the *Dbp* and *Rev-Erbα* genes (Fig. 6).

Significant reduction of *Bmal1* binding to E-box 2 of the *Dbp* and *Rev-Erbα* gene was only observed in wt but not in $TNFR1^{-/-}$ mice. Thus the reduced transcription of *Dbp* and *Rev-Erbα* is accompanied by reduced H3 histone acetylation and reduced CLOCK:BMAL1 binding at these sites.

In the experiments reported above, CD40 ligation induces SBS and leads to dysregulation of the expression of clock genes. Deletion of *Dbp* reduces locomotor activity [75], alters period length [74], and reduces consolidation of sleep episodes after sleep deprivation [75]. Therefore, we analyzed whether the extent of CD40 mAb-induced reduction of locomotor activity correlates with altered clock gene expression in the different knockout mice. As shown in Figure 7, the extent of *Dbp* suppression correlates significantly with impairment of CD40 mAb-mediated suppression of locomotor activity.

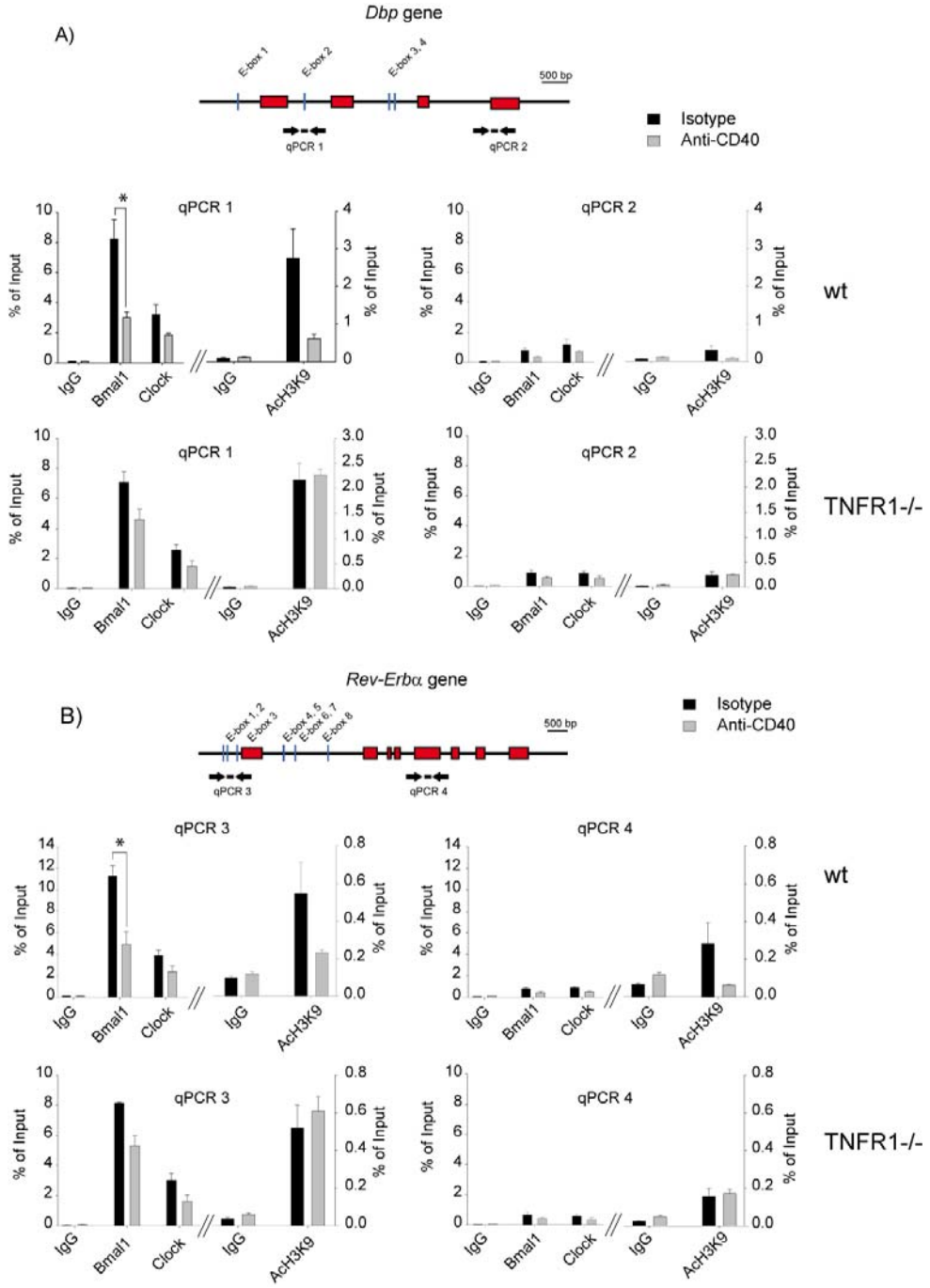


**Figure 6. CD40 ligation leads to chromatin modifications and impairsCLOCK:BMAL1 binding to E-boxes of *Dbp* and *Rev-Erbα*.** Chromatin immunoprecipitation was performed with antibodies against BMAL1, CLOCK, acetylated histone H3 lysine9 (acH3K9), and rabbit IgG. A) Schematic representation of the *Dbp* gene with E-boxes (blue), exons (red), and the quantitative PCR (qPCR) reactions. Note that pPCR 1 covers E-box 2 which has been shown to rhythmically bind CLOCK:BMAL1 complexes [103]. QPCR 2 is located in exon 4 and has no E-boxes, which serves as control. Upper panels show values of wt, lower panels of TNFR1$^{-/-}$ mice. Black bars show isotype treated, gray bars show CD40 mAb treated animals (3 mice per group). Note reduced levels of BMAL1, CLOCK and acH3K9 abundance in wt and alleviated reductions in TNFR1$^{-/-}$. B) Schematic representation of the *Rev-Erbα* gene similar to A. QPCR 3 is located near E-box 2 and qPCR 4 in exon 5. As seen with *Dbp* reductions of Bmal1, Clock and acH3K9 were less pronounced in TNFR1$^{-/-}$. Mean + s.e.m. Student's t-test. *P< 0.05.

Treatment with CD40 mAb leads to profound increase of the number of rest periods, in reduction of locomotor activity and repression of *Dbp* transcription in wt and IL-6$^{-/-}$ mice. These effects were only moderate in IL-1R1$^{-/-}$ mice as well as in heterozygous TNFRI$^{+/-}$ and MyD88$^{+/-}$ mice. Interestingly, the CD40 mAb-induced effects were not seen at all in TNFR1$^{-/-}$ and Myd88$^{-/-}$ mice. These findings indicate that CD40 ligation-mediated dysregulation of *Dbp* may be linked to the development of SBS. A trend in the same direction was also seen when correlating locomotor activity with the expression of *Rev-Erbα*, though without reaching significance. Since the latter is well known to negatively regulate the expression of *Bmal1* [101], it is interesting that the correlation of locomotor activity and *Bmal1* is found here to tend in the opposite direction compared to *Rev-Erbα*.

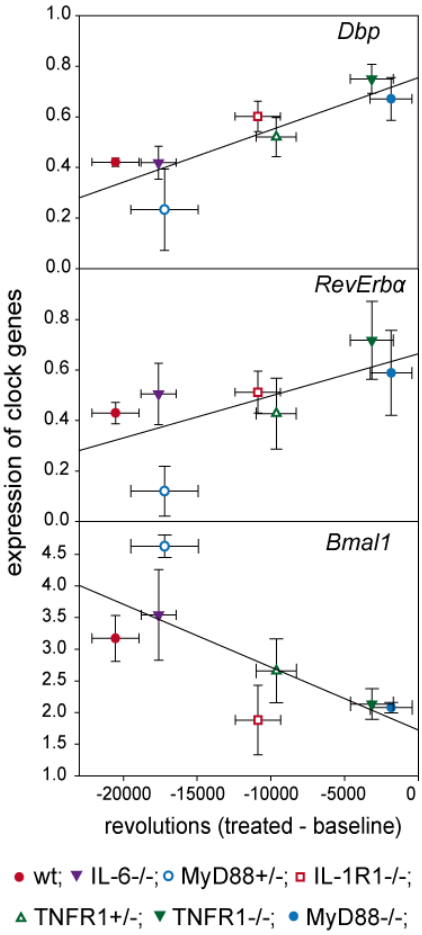


**Figure 7. Suppression of *Dbp* gene expression correlates with the degree of CD40 mAb induced locomotor activity impairment.** Pearson correlations between clock gene expression levels in liver and running wheel activity in the 12-h dark period of different gene knockout mice treated with CD40 mAb. Reduction in the expression of *Dbp* positively correlates with reduction in locomotor activity: R2 = 0.74, P = 0.01; *Rev-Erbα*: R2 = 0.43, P = 0.1. Reduction in the expression of *Bmal1* negatively correlates with reduction in locomotor activity R2 = 0.54, P = 0.059. Data are from wt (IgG2a), n = 10; wt (CD40), n = 12; IL-6$^{-/-}$ (IgG2a), n = 9; IL6$^{-/-}$(CD40), n = 11; TNFRI$^{-/-}$ (IgG2a), n = 10; TNFR1$^{-/-}$ (CD40), n = 11; TNFR1$^{+/-}$ (CD40), n = 6; IL-1R1$^{-/-}$ (IgG2a), n = 11; IL-1R1$^{-/-}$ (CD40), n = 11; MyD88$^{-/-}$ (IgG2a), n = 8; MyD88$^{-/-}$ (CD40), n = 10; MyD88$^{+/-}$ (CD40), n = 6.

## DISCUSSION

Here we found that ligation of CD40 leads to a pronounced impairment of locomotor activity and increased rest periods in mice at night, the time period the animals are usually active. Since this effect is not seen in TNFR1 deficient mice, TNF is the likely mediator of the behavioural changes. This is further substantiated by the observation that TNF injections in MyD88$^{-/-}$ mimic the effect of CD40 mAb and even revoke their resistance to develop SBS mice in response to CD40 mAb treatment. TNF serum concentrations in CD40 mAb treated MyD88 $^{-/-}$ mice were significantly lower compared to wt controls. Recent studies show that in MyD88$^{-/-}$, the response to TLR agonists is abolished and TNF production induced by IL-1β is severely diminished [104]. Ligation of CD40 and subsequent binding to tumour necrosis factor receptor – associated factors (TRAF) leads to MyD88-independent signalling pathways including NF-κB with subsequent up-regulation of proinflammatory cytokines such as TNF, IL-1β and IL-6 (for review see [96]). CD40 ligation-induced SBS depends on TNFR1, but neither on IL-1R1 nor IL-6. TNFR1 has also been reported to mediate SBS in mice treated with TNF, the effects being dependent on the TNFR1 adaptor protein FAN [105]. TLR-mediated SBS induced by LPS, as measured by immobility, social exploration, and body weight changes, still occurred in IL-1R1$^{-/-}$ mice or in mice treated with neutralizing antibodies to IL-1β, IL-6 and TNF [35, 106]. Furthermore, administration of TNF blockers failed to prevent LPS-induced SBS [107]. Taken collectively TNF in TLR-mediated SBS seems of less importance, the TNF:TNFRI interactions, however, are crucial in CD40 ligation-induced SBS. Since CD40 mAb-induced impairment of locomotor activity is less pronounced in IL-1RI$^{-/-}$ mice compared to wt mice, IL-1β is apparently less relevant than TNF to induce SBS.

In the treatment of lymphoma, chronic lymphatic leukemia and multiple myeloma, the most common adverse event of humanized, agonistic CD40 mAb (Dacetuzumab) or of CD40L expressed by a replication-deficient adenovirus, is fatigue, which developed in up to 50% of the patients [99, 108-110]. In patients with solid tumours, CD40 mAb led to a pronounced increase of IL-6 and TNF in serum [111]. A more direct link between TNF and fatigue has been provided in older studies trying to treat cancer patients with TNF. Infusions of TNF induced a state of lethargy and severe fatigue and anorexia [112-113].

Ligation of CD40 on antigen presenting cells (APC), including dendritic cells and monocytes, is important in stimulating expression of costimulatory molecules (CD80 and CD86) and production of TNF, IL-1β, IL6, IL-8, and IL-12 as well as in the rescue of circulating monocytes from apoptotic death (for review see [96]). CD40 ligation *in-vivo* induces an

inflammatory response in the lungs secondary to activation of bone-marrow-derived CD40 positive cells [114]. CD40 mAb also trigger liver inflammation which is mediated by IFNγ and TNF [115]. Our data show that mice treated with CD40 mAb develop splenomegaly and loose significant weight. The increase in spleen size was seen in wt, IL-1R1$^{-/-}$ and IL-6$^{-/-}$ mice and even developed in MyD88$^{-/-}$ and TNFR1$^{-/-}$ mice, which nevertheless, are resistant to CD40 mAb-induced SBS.

However TNFR1$^{-/-}$ mice, and even more so MyD88$^{-/-}$ mice, appear to be protected from CD40 mAb-induced weight loss. TNF is a catabolic cytokine. Transplantation of Chinese transgenic hamster ovary cells expressing the human TNF developed cachexia, with progressive wasting, anorexia, and early death [27]. TNFR1$^{-/-}$ mice injected with lung carcinoma cells showed reduced wasting compared to wt control mice, despite there being equal levels of serum TNF in both groups [116]. In experimental sepsis and tumour models chronic low level of TNF production results in sustained wasting and promotes insulin resistance [117-118]. In rheumatoid arthritis 67% of patients are diagnosed as having rheumatoid cachexia [119]. The TNF blocker Etanercept induced weight gain in at least some patients, the effect not being confirmed in another study [120-121]. Though, other factors such as IL-1β and IL-18 may contribute to rheumatoid cachexia. As seen with TNF also IL-1β administration promotes anorexia [122] and IL-18 has been found to modulate food intake and metabolism [123]. Unlike TNFR1$^{-/-}$ and MyD88$^{-/-}$ mice, IL-6$^{-/-}$ mice respond to CD40 mAb with pronounced weight loss. This contrasts with TLR-mediated changes in body weight, which after LPS injection was more pronounced in wt compared to IL-6$^{-/-}$ mice [21].

The circadian control of rest / activity cycles is provided by the hypothalamic suprachiasmatic nuclei which, by paracrine signalling, regulate circadian cycles of gene expression in every cell [65]. Administration of TNF alters the expression of clock genes, the genes which modulate circadian activity in the SCN and peripheral cells [92]. As reported for TNF, CD40 mAb also lead to suppression of *Period* and *Rev-Erbα* genes and of the clock-controlled genes *Dbp*, *Tef* and *Hlf*. The CD40 ligation-induced interference with *Rev-erbα* expression is paralleled by upregulation of *Bmal1*. This may be the consequence of the physiological role of REV-ERBα to bind to RORE DNA sequences in the *Bmal1* promoter and thereby to inhibit transcription of the *Bmal1* gene. As true for CD40 mAb-mediated impairment of locomotor activity with increased rest periods, also the CD40 ligation-induced dysregulation of clock gene expression depends on TNFR1, but not on IL-6 or IL-1R1. Recently, TNF was found to attenuate transcription of clock genes in mouse embryonic fibroblasts by interfering with the enhancing effects of DNA E-box sequences [92]. The data presented here substantiate this

finding by showing impaired BMAL1 binding to E-boxes in the respective *Dbp* and *Rev-Erbα* promoters at ZT9, the effect being seen only in nuclear liver extracts obtained from CD40 mAb treated wt, but not when derived from TNFR1$^{-/-}$ mice exposed to CD40 mAb. In addition, rhythmic histone acetylation underlies active or inactive transcription of clock genes [69]. Histones H3 are found acetylated at lysine 9 (AcH3K9) during high transcriptional activity and deacetylated during inactive transcription [103]. CD40 treatment lowered only the levels of AcH3K9 at the E-boxes of *Dbp* and *Rev-Erbα* in liver samples from wt mice, but not from TNFR1$^{-/-}$ mice. Thus transcriptional clock gene deactivation by CD40 as judged by acetylated H3 is dependent on TNF signalling. Given the link between the clock and metabolism, the alterations in the molecular clock described above may altogether contribute to the vast changes of metabolic and physiologic parameters as seen in SBS [9, 124]. TNF:TNFR1 interactions in CD40 mAb treated mice lead to increased rest periods and altered clock gene responses. These two TNF evoked responses may in fact be linked to each other. Deletions of clock genes in mice lead to altered stability, amplitude and / or period length of activity cycles (Supplementary Table 1). Thus we looked for correlations in the extent of CD40 mAb-triggered impairment of locomotor activity and dysregulation of clock genes in the different knockout mice studied. Mice with the most dramatic CD40 mAb-induced increase in rest periods (wt and IL-6$^{-/-}$ mice) also showed the most pronounced decrease of *Dbp* expression. Concordance of the two phenomena is also seen in heterozygous TNFR1$^{+/-}$ and MyD88$^{+/-}$ mice as well as in IL-1R1$^{-/-}$ mice, both effects being less pronounced. TNFR1$^{-/-}$ and Myd88$^{-/-}$ mice were resistant to CD40 mAb-induced behaviour changes and *Dbp* suppression.

In conclusion, we have demonstrated that following CD40 ligation TNF:TNFR1 interactions lead to SBS, which may result from dysregulation of clock genes. Identification of the mechanisms which hinder efficient activation of E-box mediated transcription of clock genes following TNF:TNFR1 interactions are expected to facilitate understanding of SBS in autoimmune diseases.

**ACKNOWLEDGEMENTS**

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**METHODS**

**Material.** Recombinant murine (rm) TNF-α was purchased from Peprotech (London, U.K.). The following antibodies (Abs) were used for ChIP assays: polyclonal antibodies for BMAL1 and CLOCK were prepared from immunized rabbit sera as described [125]; polyclonal acetylated histone H3K9 (ab4441) and rabbit control IgG (ab46540) were purchased from Abcam, Cambridge, UK. For Westerns we used Abs: BMAL1, CLOCK, DBP (clone 3A6; Abnova, Taiwan), REV-ERBα (#2124; Cell Signaling, Danvers, MA, USA), NF-κB p65 (sc-372; SantaCruz, CA, USA) and U2AF65 (U4758; Sigma, Saint-Louis, MO, USA).

**Mice.** C57BL/6J mice were purchased from RCC Ltd (Füllinsdorf, Switzerland). $Tnfrsf1a^{tm1Blt}$ termed $TNFR1^{-/-}$ mice were generously provided by Prof. Mathias Heikenwälder, Univ. Zurich, Switzerland; $Il6^{tm1Kopf}$ termed $IL\text{-}6^{-/-}$ mice by Prof. Pierre-Alain Clavien, University Hospital Zurich; $Il1r1^{tmImx/J}$ termed $IL\text{-}1R1^{-/-}$ by Prof. Manfred Kopf, ETH Zurich; $MyD88^{tm1Aki}$ termed $Myd88^{-/-}$ by Dr. Barbara Stecher, ETH Zurich. All strains have been backcrossed to C57BL/6J mice for at least 10 generations. For all experiments we used male mice aged between 8-12 wks. Animals were maintained under specific pathogen–free conditions. Experiments conformed to the guidelines of the Swiss Animal Protection Law and approved by the Veterinary office of Canton Zurich. Mouse experiments were performed under licenses 55/2008 according to the regulations of the Veterinary office of the Canton Zurich.

**Locomotor activity recording.** Locomotor activity was recorded as previously described [92]. Shortly, mice were housed in individual cages, equipped with a running wheel and a passive infrared sensor in a temperature-controlled sound-proof light-tight room (approximately 22°C) with food and water ad libitium. Activity recordings were based on 1 min episodes by using the Chronobiology Kit software (Stanford Software Systems, Santa Cruz, CA). Rest episodes were defined as 1 min units with zero activity. We allowed mice 10-15 days of adaptation to the light-dark cycle (light on at 0500 = ZT0; light off at 1700 = ZT12). Mice were injected i.p. at ZT5 with 200 μg anti-CD40 antibodies (clone FGK4.5 ;

<0.61 EU/mg (LAL), BioX Cell, West Lebanon, NH, USA) in 200 µl PBS or 200 µg rat IgG2a (clone 2A3; <0.23 EU/mg (LAL), BioX cell) in 200 µl PBS. Two days after injection mice were weighted and killed at ZT 9-10. Tissues were excised and frozen until subsequent RNA extraction, or used fresh for liver nuclei isolation.

**Implantation of osmotic minipumps.** Osmotic minipumps were implanted on the back of the mice as described before [92]. Briefly, Alzet minipumps (Model 1007D; Alzet, Cupertino, CA) filled with TNF-α (1.5 µg/day, diluted in 0.1% BSA/PBS) or 0.1%BSA/PBS as a control were inserted below the skin on the back. Locomotor activity was recorded as described above. Three days after minipump insertion mice were sacrificed and organs were processed for blood or qPCR analysis.

**RNA isolation and gene expression analysis.** Mouse tissues were homogenized in peqGOLD RNA pure (PeqLab, Erlangen, Germany) and the RNA was extracted according to the manufacturer's instructions. Isolated RNA was DNA digested and purified with spin columns NucleoSpin RNA II (Macherey-Nagel, Düren, Germany). Then cDNA was synthesized using random hexamers (Applied Biosystems, AB, Rotkreuz, Switzerland) and M-MuLV reverse transcriptase (AB). The cDNA equivalent to 20 ng of total RNA was PCR-amplified in an ABI PRISM HT7900 detection system (AB) using the TaqMan Universal PCR Master Mix (AB) and quantified as previously described [126]. All samples were run in duplicate and results were normalized to 18S rRNA (AB). Relative mRNA levels are expressed as x-fold variations compared with the control treated group or as percent expression with the highest expressor set to 100 % in Fig. 4 and Supplementary Fig 6.

**Chromatin Immunoprecipitations (ChIP).** ChIP assays were performed according to Ripperger et al [103]. The DNA was isolated using the iPure kit (Diagenode) based on magnetic DNA purification. The efficiency of ChIP of the loci analyzed were calculated from qPCR data and reported as a percentage of starting material: % (ChIP / total input) = 2 ^ (($Ct_{Input}$ - (LOG(50;2)) - $Ct_{ChIP}$) * 100. Primers for genomic loci were as follows. *QPCR1* (mDbpI1), forward and reverse primers: 5'-ATGCTCACACGGTGCAGACA-3' and 5'-CTGCTCAGGCACATTCCTCAT-3', probe: 5'FAM-CCTAGTTTCCATGTGACCCTGC GAGG- 3'BHQ1. *QPCR2* (mDbpE4), forward and reverse primers: 5'-AAGAACAATGAAG CAGCCAAGAG-3' and 5'-GGCAGCCCGCACAGATAT-3', probe: 5'FAM-TGGTTCTCC TTGAGTCTTCTTGCATCTCTCG-3'BHQ1. *QPCR3* (mRev5'U), forward and reverse primers: 5'-CCCACCCCAGTCCACTTTC-3' and 5'-GCCTATT CCTTTTCTGCTTTGC-3', probe: 5'FAM-CTCCTCCCCGCTGCCAGCAA-3'BHQ1. *QPCR4* (mRevE5), forward and

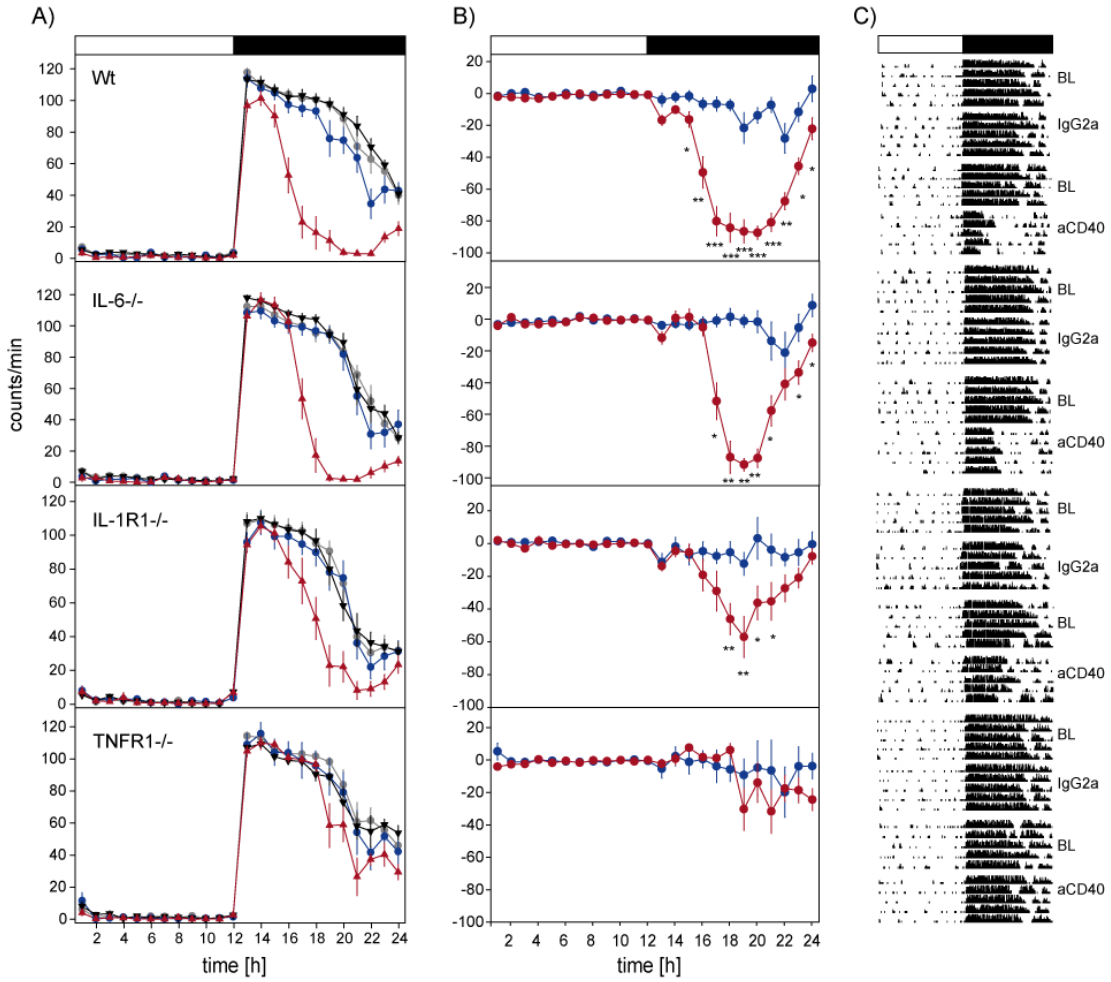
reverse primers: 5'-TCTTCACCTATGCCCATGACAA-3' and 5' GACGGGCATCC CTGA CTCT-3', probe: 5'FAM-TGCCAATCATGCAT CAGGTAGCCCTT-3'BHQ1.

**Western blot from liver nuclei.** Liver nuclei were isolated as for the ChIP assay without adding of formaldehyde. Nuclei were resuspended in NUN buffer (600 mM NaCl, 2 M urea, 2% NP-40, 50 mM Hepes-pH7.6, 2 mM DTT) and the protein concentration from the cleared lysate was measured by BCA assay (Perbio). 20 µg protein was run on a SDS gel (4-20%, Novex, Invitrogen) and blotted afterwards on PDVF filter. The blots were blocked with 5% skim milk in TBS-T, and incubated in the same buffer with antibodies overnight at 4°C. After staining with secondary antibodies the blots were developed with ECLplus (GEhealthcare, Glattbrugg, Switzerland) and exposed in a ImageQuant 350 imaging system (GEhealthcare) by using the appropriate quantitation software.

**Elisa and Luminex.** Blood was collected in Microtainer SST tubes (BD, Franklin Lakes, NJ, USA) and blood serum was obtained after short centrifugation. Sera were analyzed by luminex technology at Cytolab, Dällikon, Switzerland, or by conventional ELISA for IL-1β (Invitrogen)

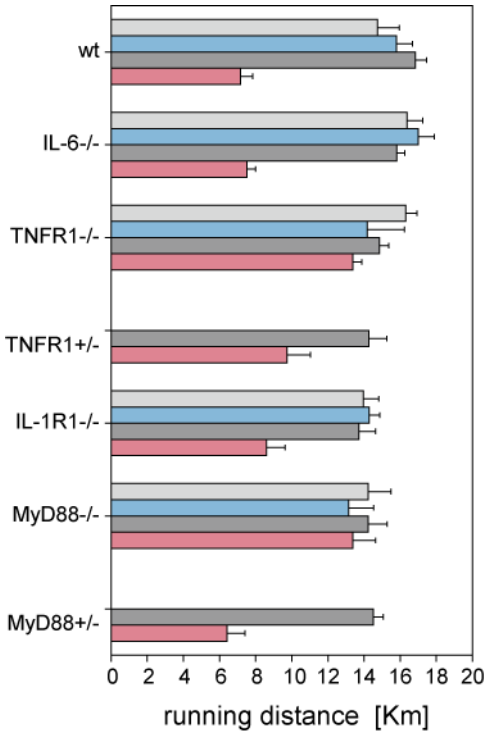
**Statistical analysis.** The comparison of running wheel and infrared activity between anti-CD40 and control treated animals was performed by two-way analysis of variance (ANOVA) with factor ‘interval’ and ‘treatment’. Whenever significant effects were present ($P \leq 0.05$) two-tailed paired t-tests were used to further evaluate differences. SAS (SAS Institute, Inc., Cary, NC, USA) and MATLAB (The Math Works, Inc., Natick, MA) were used for statistical analysis.

## SUPPORTING INFORMATION

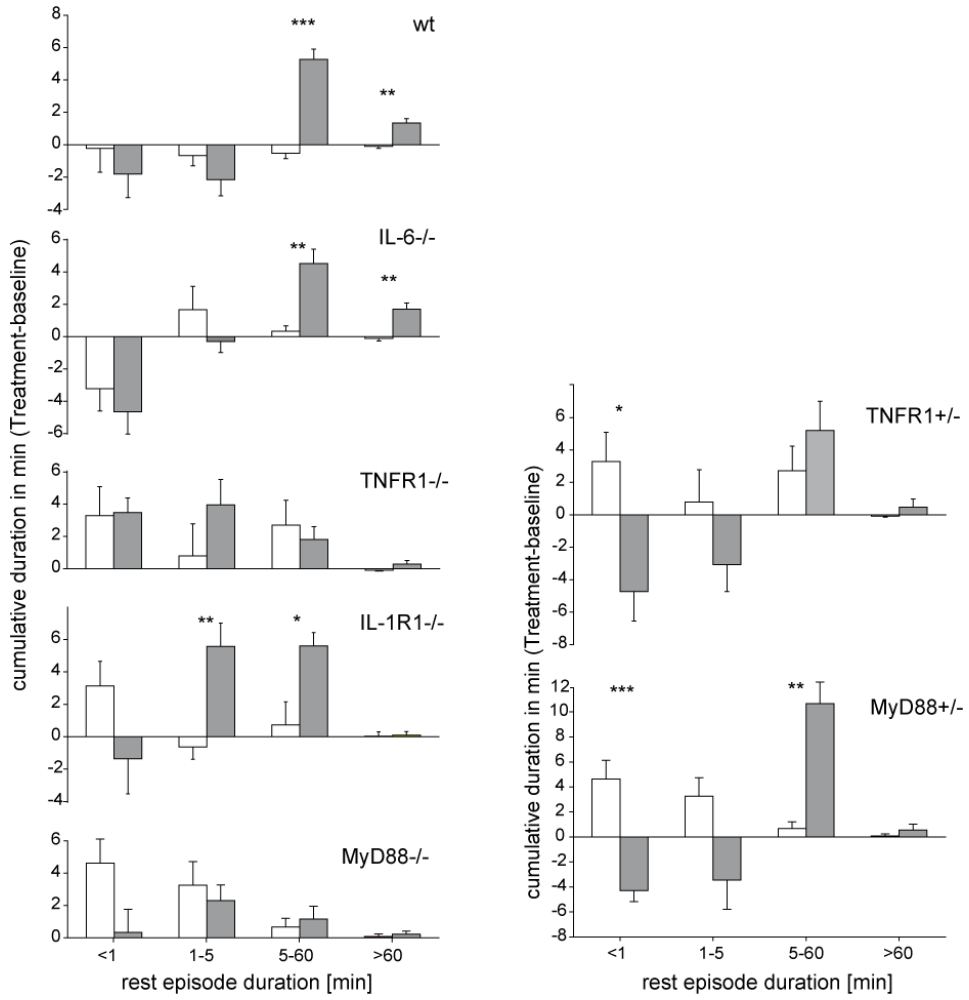



**Supplementary Fig. 1.** Intraperitoneal challenge with anti-CD40 does not affect locomotor activity in TNFRI$^{-/-}$ C57BL/6 mice. A) Infrared activity for the first day after i.p. injection of 200 µg CD40 mAb (red) or IgG2a as control (blue). Baselines are the mean of three days preceding injection of CD40 mAb (black) or IgG2a control (gray). Mean of 1-h values ± s.e.m. B) Difference in infrared activity after subtracting the baseline. Blue line: IgG2a; Red line: CD40 mAb. The data are shown as means ± s.e.m. Stars indicate 1-h interval which differed significantly between treated and baseline groups: *P<0.05, **P<0.005, ***P<0.0005, 2-tailed t-test after significant 2-way ANOVA for 'interval' and 'treatment'.

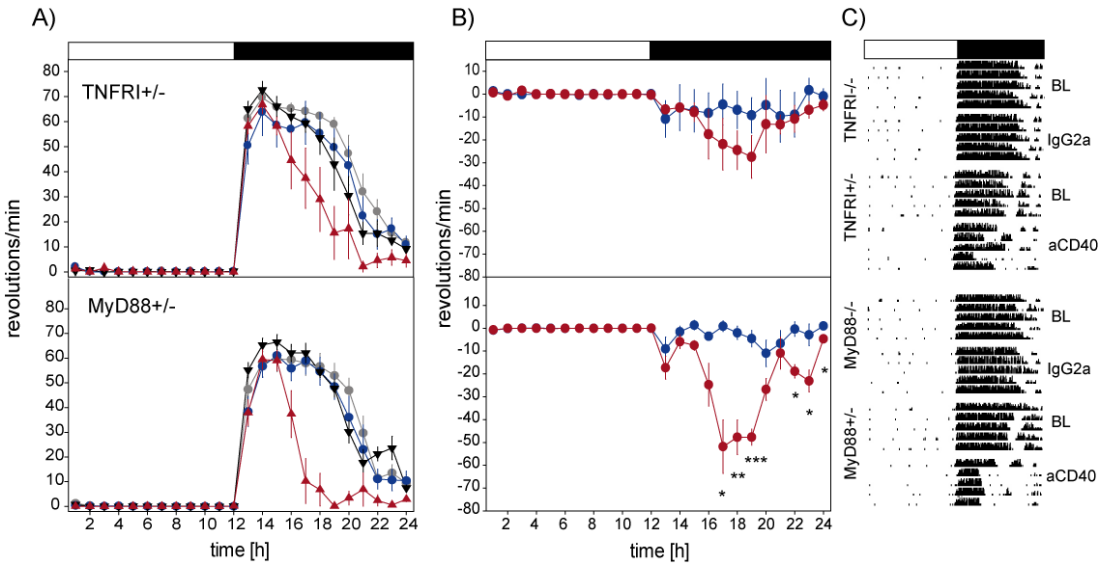
C) 24-h actograms of infrared activity of five representative mice after antibody injections. Each line represents one individual mouse, one day before (baseline, BL) and one day after injection of IgG2a and CD40 mAb. The light-dark period is indicated by the black and white bars at the top of the plot. Data are from wt (IgG2a), n = 10; wt(CD40 mAb), n = 12; IL-6$^{-/-}$ (IgG2a), n = 9; IL6$^{-/-}$ (CD40 mAb), n = 11; TNFRI$^{-/-}$ (IgG2a), n = 10; TNFR1$^{-/-}$ (CD40 mAb), n = 11; IL-1R1$^{-/-}$ (IgG2a), n = 11; IL-1R1$^{-/-}$ (CD40 mAb), n = 11; for A and B.



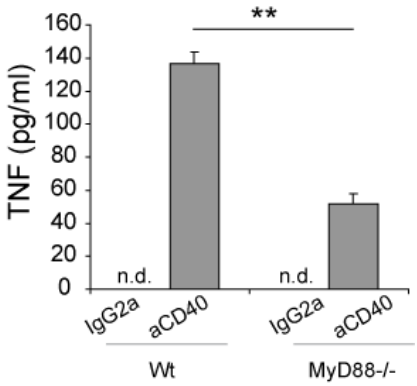

**Supplementary Fig. 2.** Running distance in the dark period prior to IgG2a and CD40 mAb treatment are shown in light and dark gray bars resp. and 24 hr after injection of IgG2a (blue bars) and CD40 mAb (red bars).



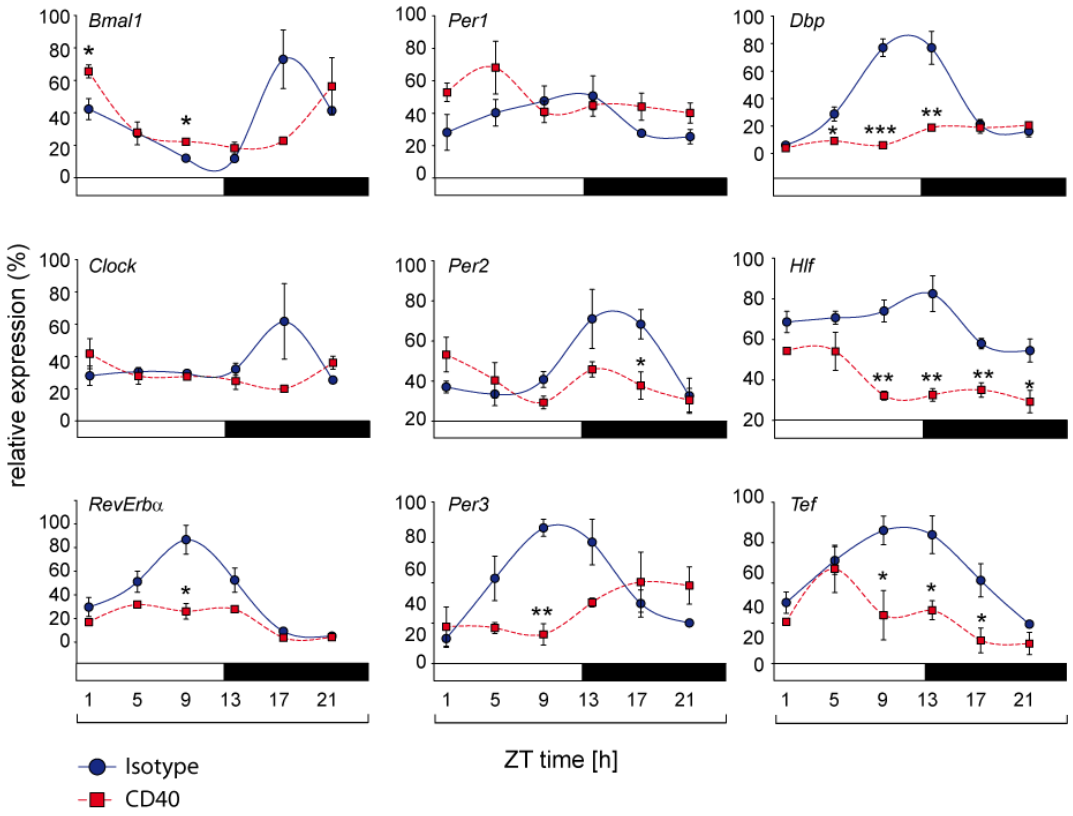

**Supplementary Fig. 3.** Mice treated with CD40 mAb show increased long rest epochs in the dark phase. Analysis of rest epochs for the 12-hr dark period one day after injection of IgG2a (white bars) or CD40 mAb (grey bars). The data show the differences in numbers of 1-min episodes with activity = 0 after having subtracted the baseline (mean of the three days before injection). The occurrence of rest was arbitrarily subdivided in episodes for durations with rest = 0 activity counts: up to 1 min rest, between 2 and 5 min, 6 and 60 min, and more than 60 min. ANOVA for repeated measures, followed by independent-samples *t*-test: * P<0.05, ** P<0.005, *** P<0.0005.



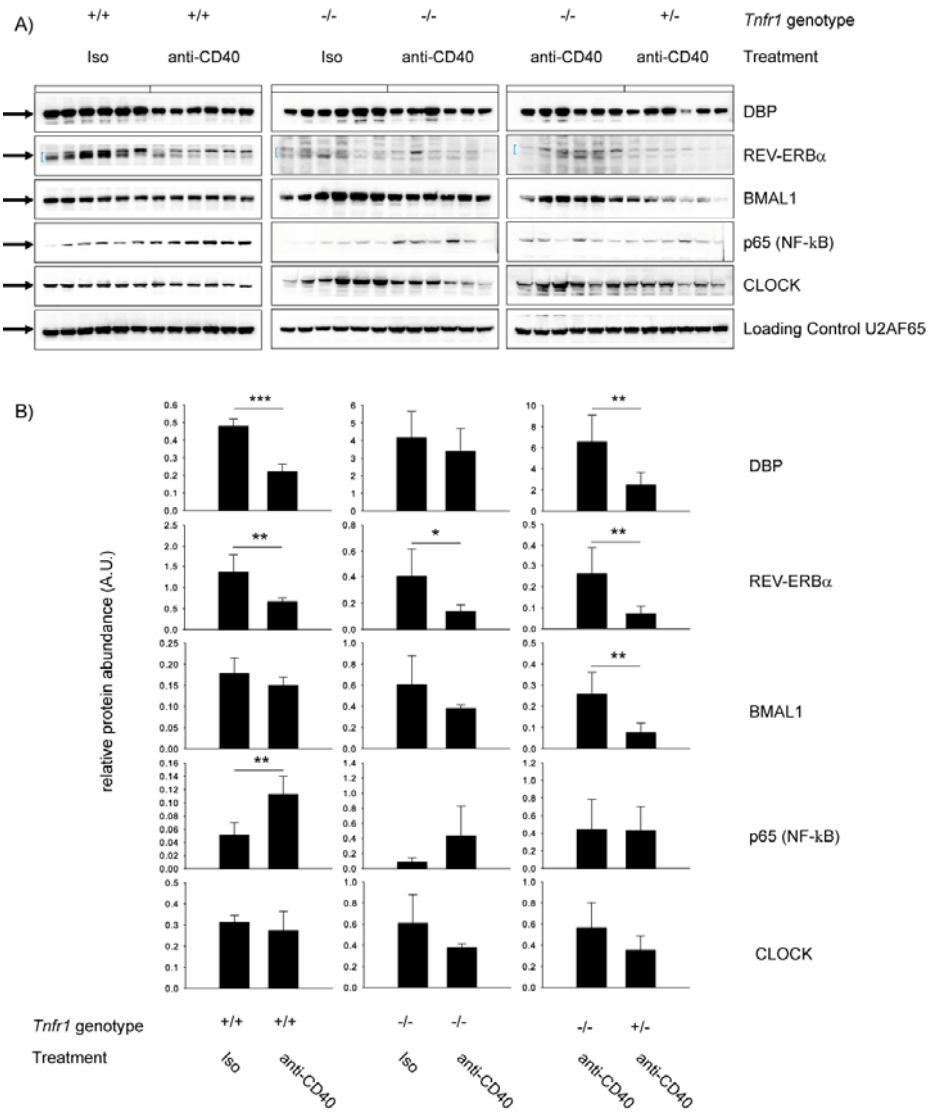

**Supplementary Fig. 4.** Effect of CD40 ligation on locomotor activity of TNFRI$^{+/-}$ and MyD88$^{+/-}$ mice. Running wheel activity for the first day after i.p. injection of 200 µg CD40 mAb or IgG2a as control. A) Frequency of running wheel activity for the first day after i.p. injection of 200 µg CD40 mAb (red line) or IgG2a as control (blue line). Baselines are the mean of three days preceding injection of CD40 mAb (black line) or IgG2a control (gray line). Mean of 1-h values ± s.e.m. B) Difference in running wheel activity after subtracting the baseline. Blue line: IgG2a; Red line: CD40 mAb. The data are shown as means ± s.e.m. Stars indicate 1-h interval which differed significantly between treated and baseline groups: *P<0.05, **P<0.005, ***P<0.0005, 2-tailed t-test after significant 2-way ANOVA for 'interval' and 'treatment'. C) Actograms show running wheel data from five representative mice after injection. Each line represents one individual mouse, one day before (baseline, BL) and 1 day after injection (IgG2a and anti-CD40 resp.). The LD period is indicated by the black and white bars at the top of the plot. TNFRI$^{+/-}$: n = 6; TNFRI$^{-/-}$: n = 10; MyD88$^{+/-}$: n = 6; TNFRI$^{-/-}$: n = 10; MyD88$^{-/-}$: n = 10.




**Supplementary Fig. 5.** A) Low serum TNF concentrates in MyD88$^{-/-}$ mice. TNF was measured by ELISA in serum of wt or MyD88$^{-/-}$ two days after injection of CD40 mAb or IgG2a control antibodies. Means ± s.e.m. **P<0.005, Mann-Whitney test. B) Serum cytokine levels one day after anti-CD40 treatment or IgG2a measured by Luminex, TNF and IL-6, and ELISA IL-1β.




**Supplementary Fig. 6.** Circadian gene expression of clock and clock-controlled genes are impaired by anti-CD40 treatment in the kidney. During a 24 h time period (one day p.i.) gene expression was quantified by qPCR for the respective genes (compared to 18s rRNA endogenous control and calibrated to the highest expressor, i.e. 100% expression). Note induced levels of *Bmal1* at ZT9, and profound reductions in the amplitude expressions of E-box dependent clock, i.e. *Rev-Erbα*, *Per2*, *Per3*, and clock-controlled genes, i.e. *Dbp*, *Hlf*, and *Tef*. Three mice per time point and treatment group were analyzed. Mean ± s.e.m. 2-tailed Student's t-test: *P<0.05, **P<0.005, ***P<0.0005.




**Supplementary Fig. 7.** The abundance of clock proteins in liver nuclear extracts is impaired by anti-CD40 and reveals differences between wt and TNFR1$^{-/-}$ mice. A) Western blots of different clock proteins from liver nuclear extracts from individual mice (each lane) with different *tnfr1* genotypes (+/+, -/-, +/-). DBP (40 kDa), REV-ERBα (82 kDa), BMAL1 (80 kDa), CLOCK (105 kDa), NF-κB-p65 (65 kDa), and nuclear expressed U2AF65 (65 kDa) endogenous control were immunostained by conventional western blot technique. B) Densitometric quantification of western blots shown in A. The relative protein abundance is calculated by the ratio of the clock protein to endogenous control. Note significant downregulations of DBP and REV-ERBα proteins and upregulation of p65 in anti-CD40 treated wt mice (left panels). In contrary, TNFR1$^{-/-}$ mice (middle panels) show less significant impairments of clock proteins. Significant differences in DBP, REV-ERBα, and BMAL1 were also detectable when comparing anti-CD40 treated homozygous vs. heterozygous knockouts. downregulation of REV-ERBa and only minor changes a  from six different mice per treatment group. Left panel shows wt mice, middle panel TNFR1-/- mice, and right panel compares knockouts with heterozygous mice. Three mice per time point and treatment group were analyzed. Mean ± s.e.m. 2-tailed Student's t-test: * P<0.05, ** P<0.005, *** P<0.0005.

## 4.2 Clock gene modulation by TNF depends on calcium and p38 MAP kinase signalling

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**ABSTRACT**

Treatment of fibroblasts for 24 hours with tumour necrosis factor-alpha (TNF) suppresses transcription of E-box driven clock genes (*Dbp*, *Tef, Hlf* and *Per1*, *Per2*, *Per3*) by yet unknown molecular mechanisms. The attenuation of clock genes has been suggested as a putative cause for the development of sickness behaviour syndrome in infections and autoimmune diseases. Here, we studied the effect of TNF-α at early time points (<3 h) on intracellular signalling events and clock gene expression in fibroblasts. Interaction of TNF with TNFR1 (Tnfrsf1a, CD120a, p55) leads to downregulation of gene expression of D-site albumin binding protein (*Dbp*) and upregulation of negative regulators of the molecular clock, *Period-1* and *-2* (*Per1* and *-2*), *Cryptochrome-1* (*Cry1*), and *Differentiated embryo chondrocytes-1* (*Dec1*). Since the decrease of *Dbp* is also observed in knockout cells for *Per1/2* and *Dec1*, these genes are unlikely to be responsible for inhibition of *Dbp.* The effect of TNF on the clock genes, *Dbp* and *Per1*, in the early phase is dependent on p38 mitogen activated protein kinase (MAPK) and/or calcium signalling but not on the activation of NF-κB or AP-1. Taken collectively the data show p38 MAPK and calcium dependent, TNFR1 mediated transient increase of negative regulators of the molecular clock and an independent decrease of *Dbp*.

## INTRODUCTION

TNF is a pleiotropic cytokine, which regulates the activation and function of a variety of cells including lymphocytes, fibroblasts and neurons [128]. Its receptors, especially TNFR1, are ubiquitously expressed [129]. In response to infections or in inflammation during autoimmune diseases (e.g. rheumatoid arthritis, multiple sclerosis, and inflammatory bowel disease) TNF is produced mainly by dendritic cells and macrophages [128]. While infusions of TNF in cancer patients lead to severe daytime fatigue [112], treatment of patients with rheumatoid arthritis or obstructive sleep apnoea syndrome with TNF blockers reduces disease-associated fatigue [130-131]. On the other hand, a disruption of the circadian clock can lead to the same symptoms. These data indicate a possible influence of TNF on the circadian system and thereby on fatigue.

Recently, the effect of TNF on the molecular clock was investigated by our group. The data showed that TNF reduces the expression of E-box driven clock genes in cultured fibroblasts. Moreover TNF impairs nocturnal locomotor activity in mice, the effect being associated with a decrease of clock genes in the liver [92]. These results suggest that TNF, by interfering with clock gene expression, might attenuate the circadian output of physiological and behavioural rhythms. Here, we aimed to elucidate signalling events leading to reduced clock gene expression, in TNF treated fibroblasts.

The molecular clock is the cellular basis of circadian rhythms found in almost every organism ranging from bacteria, plants, and flies to humans. Circadian rhythms control many aspects of physiology and metabolism that allow organisms to anticipate regular changes in the environment (for reviews see [64]). In mammals, the integration of the day and night variation is provided by the suprachiasmatic nucleus (SCN) of the hypothalamus, which receives light information from the retina and synchronizes the clock of other brain areas and peripheral organs. Rhythms in single cells are thought to origin from interlocked transcription-translation feedback loops composed of positive transcriptional activators *Clock* and *Bmal1* and negative elements such as *Per* and *Cry*. CLOCK and BMAL1 heterodimerize and bind to E-box enhancer sequences to promote transcription of *Per1*, *Per2*, *Cry1*, and *Cry2*, the products of which inhibit the activity of CLOCK:BMAL1 and hence their own transcription. A second feedback loop controls the expression of the positive clock element: REV-ERBα - itself activated through E-boxes by CLOCK:BMAL1 - rhythmically inhibits *Bmal1* transcription [132]. Post-translational modifications, such as phosphorylations, acetylations, sumoylations, among others allow for the fine tuning of the system generating a period of about 24 hours. Clock-controlled genes (CCGs) such as *Per3* and the members of the PAR-bZip family of

transcription factors, *Dbp*, *Tef*, and *Hlf*, also bear E-box motifs and their periodical expression contributes to the transformation of time information into physiological and behavioural rhythms. However, they are not integral part of the timing system.

TNF signals via two receptors: TNFR1 (p55TNFR, CD120a, TNFRSF1a) and TNFR2 (p75TNFR, CD120b, TNFRSF1b). Whereas TNFR1 is ubiquitously expressed and more effective in sensing soluble TNF; TNFR2 has a higher affinity to the membrane bound cytokine, is involved in ligand passing to TNFR1 and bears its main functions in chronic inflammatory conditions. After receptor activation different intracellular adaptor proteins direct a complex array of signalling processes mediated by kinases, phosphatases, reactive oxygen intermediates, lipases, caspases, and sphingomyelinases. This leads to regulation of the cell function by transcription factors, G-protein signalling, and calcium ion signalling. In the TNF dependent intracellular signalling cascade, activation of caspases and the transcription factors NF-κB and AP-1 are important steps and have been found to regulate apoptosis and expression of chemokines and cytokines.

This study was aimed to characterize the relevant intermediates of TNF signalling leading to altered clock gene expression. In the early response of fibroblasts to TNF, transcription of negative clock gene regulators, namely *Per1/2*, *Cry* and *Dec1* is activated. However, this induction is unlikely to be involved in downregulation of the clock-controlled gene *Dbp.* Impaired expression of *Dbp* was not dependent on caspase signalling, NF-κB, or AP-1 activation, but rather on the calcium ion signalling. Although there is less binding of acetylated histone H3 to the *Dbp* E-box 857, active deacetylation is not involved in the inhibition of *Dbp* by TNF.

## RESULTS

### Immediate early induction of negative clock elements parallels fast *Dbp* downregulation.

To elucidate the direct effects of TNF on clock gene expression, we followed the immediate early response in NIH 3T3 fibroblasts. Upon treatment with TNF-α, expression of the clock controlled gene *Dbp* started to decrease after 15 min and reached 20% of control after 2 h (Fig. 1A, top left). A more delayed downregulation was observed when analyzing the expression of *Per3* (Fig. 1A bottom middle). In contrast, *Per1* - and to a lower extent also *Per2* - showed a significant transient peak of expression at 1 h after stimulation (Fig. 1A and B), with subsequent downregulation at later time points. Whereas both *Cry1* and *Dec1* were successively upregulated, the effect of TNF on *Cry2* and *Dec2* was less pronounced and the expression of *Clock* was hardly affected (Fig. 1A). The *Per1* gene is regulated by two alternative promoters [133] regulating two alternative first exons, i.e. 1A and 1B.

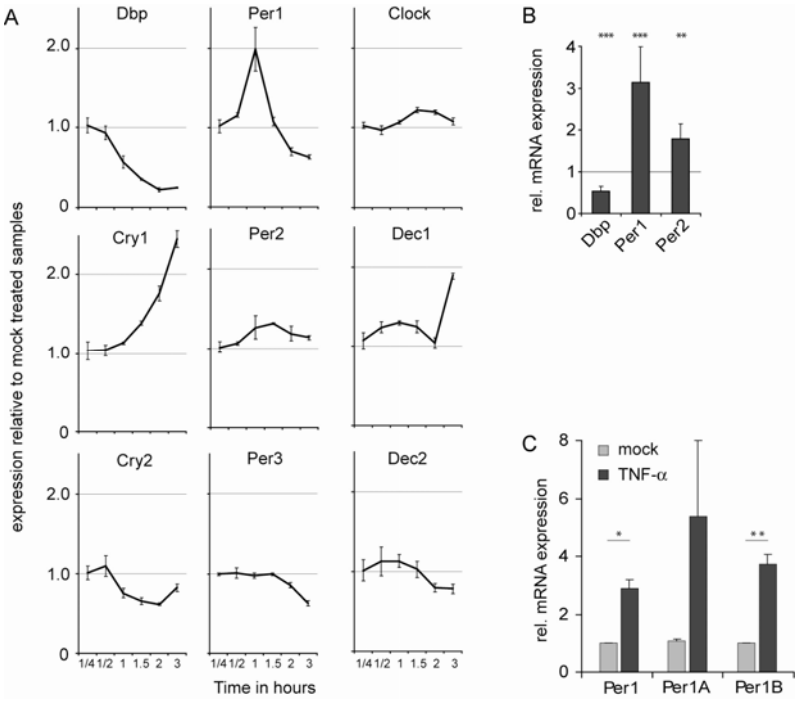



**Fig. 1: Early expression time course of clock genes in TNF treated NIH 3T3 fibroblasts.** (A) Cells were stimulated with 10 ng/ml TNF in serum-free medium and mRNA expression of clock genes was analyzed at the indicated time points. Shown is the mean ± SD from 1 experiment in triplicates out of 2. (B) *Per1* and *Per2* are significantly induced and *Dbp* significantly inhibited after 1 h stimulation with TNF-α. Shown is the mean + SD; n = 10 for *Per1* and *Dbp*, n = 6 for *Per2*. (C) Induction of *Per1* is mediated by promoter 1A and 1B. NIH 3T3 were stimulated with 10 ng/ml TNF-α for 1 h; n = 3 in triplicates. Shown is the mean + SD. For B and C we used paired sample t-tests: *P< 0.05, **P< 0.005, ***P< 0.0005

There was no difference in the *Per1* peak induction at 1 h at the different promoters indicating similar regulation of both promoters (Fig. 1C). Taken collectively, the data show that the clock genes analyzed respond differently to TNF treatment in a time dependent manner.
In further experiments we tested if TNF induces increased degradation of *Dbp* mRNA. When blocking RNA transcription with Actinomycin D, no enhanced degradation of the short-lived *Dbp* mRNA was observed (Fig. S1A). However, we still found downregulation of pre-mRNA transcription by analysis of nascent transcripts (Fig. S1B). Hence, the effect of TNF to downregulate *Dbp* mRNA is due to a block in transcription and to the short lifespan of the mRNA.

**Cycloheximide partly de-represses *Dbp* inhibition**

To assess the dependency of *Dbp* downregulation on *de-novo* induced proteins, we performed assays with cycloheximide (CHX), a compound inhibiting protein biosynthesis. Five hours before TNF treatment, CHX was added to the cell cultures to inhibit protein translation. Thereafter, TNF was added only for 2 h because a longer exposure was found to induce apoptosis. The inhibition of *Dbp* expression was reversed but could not be de-repressed completely (Fig. 2).

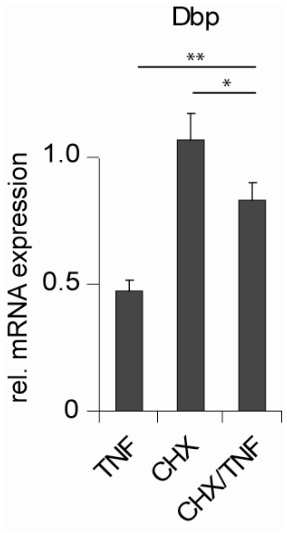



**Fig. 2: The inhibition of *Dbp* is partly dependent on *de-novo* protein synthesis**. After a 5 h pretreatment with cycloheximide (100 µg/ml) NIH 3T3 fibroblasts were stimulated with TNF for 1 h. Expression of untreated samples corresponds to 1. Shown is the mean + SD. n = 3 in triplicates; paired samples t-test: *P< 0.05, **P< 0.005.

These results show that the downregulation of *Dbp* is partly dependent on *de-novo* protein synthesis e.g. of a short-lived protein, but cannot be fully explained by a newly produced putative inhibitor, a fact also supported by the fast kinetics of *Dbp* downregulation.

**The transient induction of negative regulators by TNF is not involved in later *Dbp* repression**

Mammalian *Per* genes are necessary for the generation of proper circadian rhythm. The induction of *Per1* which acts as a negative feedback regulator could be important for the long-lasting TNF-α mediated downregulation of clock genes. Increased *Per* gene expression could lead to increased PER levels with putatively enhanced stability and activity by posttranscriptional modifications. To evaluate this hypothesis, we analyzed mouse embryonic fibroblasts (MEFs) lacking *Per1*, *Per2*, or both of them [134]; those were compared to MEFs from corresponding wt mice. The baseline expression of the clock genes, *Dbp*, *Clock*, and *Bmal1* is strongly reduced in *Per1*$^{-/-}$ and lower in *Per2*$^{-/-}$ MEFs. For unknown reasons the expression of *Dbp* in *Per1/2*$^{-/-}$ was not different compared to wt MEFs (Fig. 3A, left panel). However, in regard to the TNF-α response it becomes clear that the ability of TNF to downregulate *Dbp* gene expression was still present in all genotypes when compared to baseline levels (Fig. 3A, right panel). Only a moderate loss of effect of TNF was seen in *Per1*$^{-/-}$ and *Per2*$^{-/-}$ MEFs cells, which however was not seen in double mutant cells. A faint upregulation of *Clock* and *Bmal1* was observed in all genotypes upon TNF challenge (Fig. 3A right panel). In conclusion, the mutations of *Per* genes had no major impact on the effects of TNF on the clock genes analyzed.

We also analyzed whether the TNF induced increase of *Dec1* (Stra13/Sharp2/BHLHB2) is responsible for downregulation of *Dbp* (Fig 1A). This possibility rose because of recent data on DEC1 to impair E-box mediated transcription [135]. Our data show that TNF effectively repressed *Dbp* gene transcription also in the human keratinocyte cell line, HaCaT. However, this effect was not de-repressed in *Dec1*-deficent HaCaT cells which have a targeted inactivation of *Dec1* on both alleles through homologous recombination (Fig. 3B) [136]. Taken collectively, our data show that despite of being upregulated at early time points neither PER1 and PER2 nor DEC1 seem to act as *Dbp* repressor molecules in TNF-α treated cells.

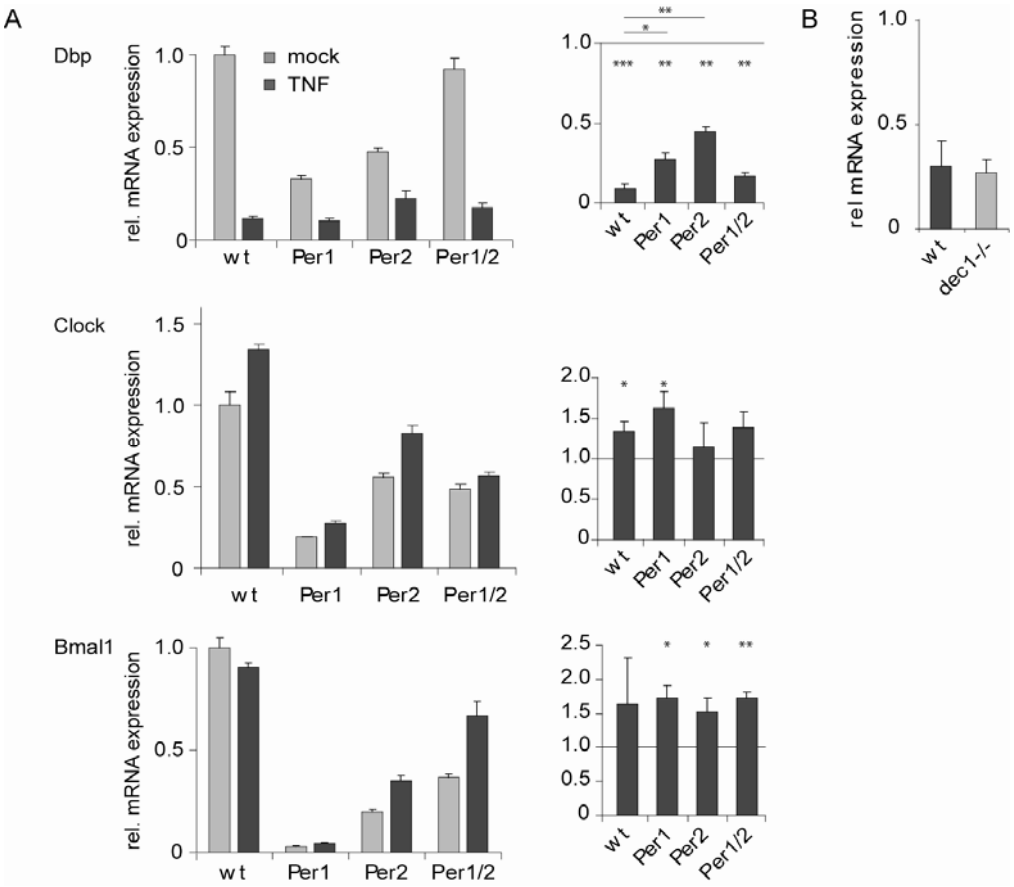



**Fig. 3: The transient induction of negative clock regulators by TNF is not responsible for the repression of *Dbp*.** (A) *Dbp* expression is repressed in wt, *Per1*, *Per2* or *Per1/2* double mutant MEFs, whereas the expression of the central clock genes *Clock* and *Bmal1* remains unchanged. Cells were stimulated with 10 ng/ml TNF for 6 h. Shown is the mean + SD; left panel shows 1 representative experiment done in triplicates where the gene expression in wt untreated cells is set to 1; right panel is the mean of 3 independent experiments done in triplicates, where 1 represents the relative expression in the corresponding not TNF treated MEFs. (B) *Dbp* inhibition is independent of the presence of DEC1. Human keratinocytes, HaCaT cells, lacking the *Dec1* gene were treated with 10 ng/ml TNF for 4 h. Shown is the relative expression compared to untreated cells. Bars represent mean + SD. n = 3 in triplicates. For A and B we used paired samples t-test: *P< 0.05, **P< 0.005, ***P< 0.0005.

## Signalling via TNFR1 leads to the *Per1* peak and *Dbp* downregulation independent of NF-κB, AP-1 or caspase activation

To analyze the dependency of TNF receptors on the clock gene responses - i.e. *Dbp* downregulation and *Per1* peak - we generated cells from TNFR1$^{-/-}$, TNFR2$^{-/-}$, and wt mice. Using RT-PCR, TNFR1 mRNA expression was found to be abolished in TNFR1$^{-/-}$ but still present in TNFR2$^{-/-}$ MEFs. TNFR2 mRNA expression is detected in both knockout genotypes, because the *tnfr2* deletion is situated outside of the amplified sequence (Fig. 4A).

However, cell surface receptor expression is completely abolished in the respective knockouts (Fig. 4B). After 1 h TNF stimulation, the *Per1* peak could be detected in wt MEFs similar, although to a lower extent than in NIH 3T3 cells. A *Per1* peak was also seen in TNFR2$^{-/-}$, but not anymore in the TNFR1$^{-/-}$ MEFs (Fig. 4C, left panel). To assay the downregulation of clock-controlled genes we looked at a later time point (6h), where inhibition is complete for all genes usually affected [92]. *Dbp* and *Per3* were downregulated in wt and TNFR2$^{-/-}$, but not in the TNFR1$^{-/-}$ MEFs, whereas *Clock* and *Per1* were unaffected in all genotypes (Fig. 4C, right panel). However, it is to note that, for unknown reasons, *Per1* was not downregulated in wt MEFs which is in contrast to the strong inhibition seen in NIH 3T3 cells. Our data show that both the TNF induced *Per1* peak at early time points and the inhibition of *Dbp* is mediated by TNFR1.

The major signalling pathway induced by TNFR1 leads to the activation of NF-κB transcription factor by its deliberation from the complex with the inhibitor of κB (IκB) proteins and subsequent translocation to the nucleus. The NF-κB transcription factor is composed mainly of the dimerized partners p65 and p50, transcriptional activity being strictly dependent on p65. Targeted inactivation of the RelA (p65) subunit of NF-κB in mice showed embryonic lethality due to massive liver apoptosis [137]. The lethal phenotype could be reversed by breeding the mice in TNF deficient animals [138]. Here, we analyzed p65$^{-/-}$ MEFs compared to wt control MEFs of the same genotype. As expected in western blot of p65, p65$^{-/-}$ clearly distinguish from wt MEFs (Fig. 5A, left panel). Further, we analyzed the capability of these cells to activate a 4x-κB site element luciferase reporter gene and IL-6 induction. The p65$^{-/-}$ MEFs had lower luciferase baseline levels and were not able to induce NF-κB after short TNF treatment (data not shown). In addition, p65$^{-/-}$ MEFs lacked the ability to respond to TNF by inducing IL-6 mRNA (Fig. 5A, right panel). Since TNF treatment in p65$^{-/-}$ MEFs leads to apoptosis starting around 4 h we analyzed the effects at time points 1 h (*Per1* peak) and 4 h (*Dbp* repression). In p65$^{-/-}$ MEFs TNF still induced the *Per1* peak and repressed *Dbp* expression (Fig. 5B). This observation is supported by lack of effect on *Dbp* inhibition when adding wedelolactone (data not shown). This compound inhibits NF-κB-mediated gene transcription by blocking the phosphorylation and degradation of IκB.

In addition to NF-κB activation, we also used an inhibitor to the c-jun N-terminal kinase (JNK), which phosphorylates c-Jun. Together with c-Fos, c-Jun forms the transcription factor AP-1. Again, early effects on *Per1* peak and *Dbp* repression at 1 h were unaffected by the inhibition (Fig. 5C). The same holds true for inhibition of caspases with a pan-caspase inhibitor (Fig. S2).

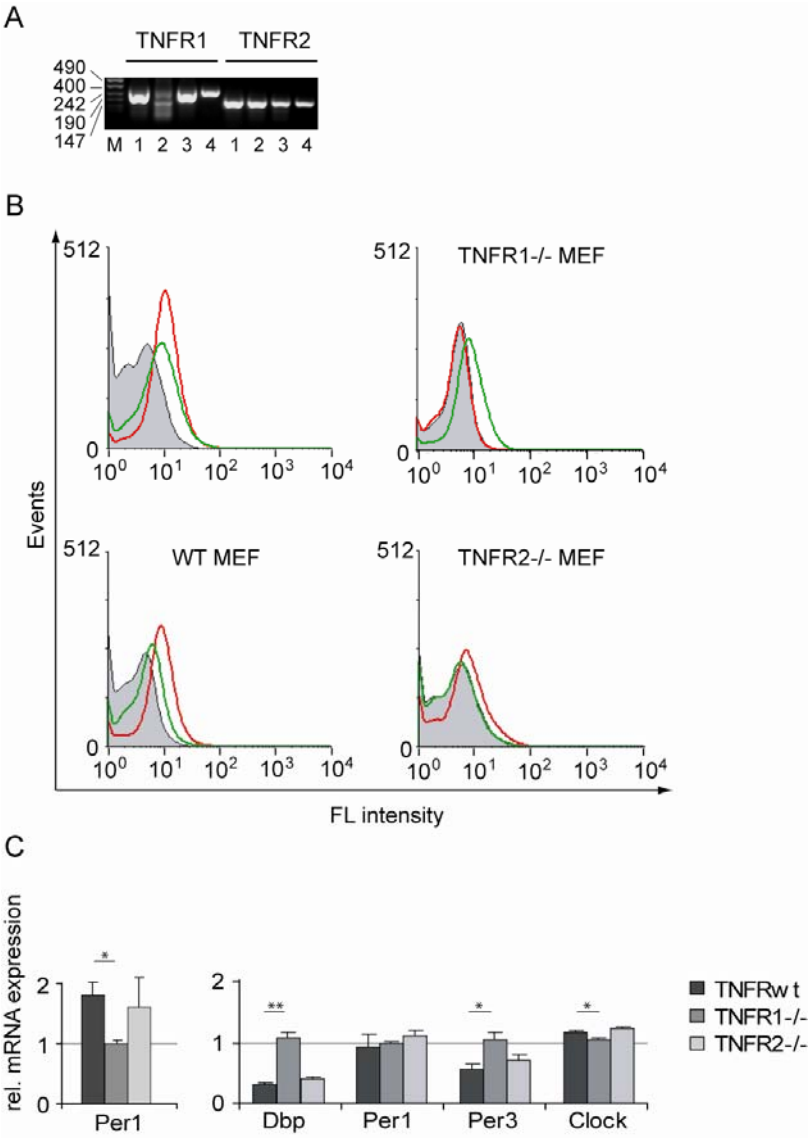



**Fig. 4: TNFR1-dependent *Per1* induction and *Dbp* repression.** (A) RT-PCR of TNFR1 and TNFR2 in MEFs from wt (1), TNFR1$^{-/-}$ (2), TNFR2$^{-/-}$ (3), or peritoneal macrophages (4). (B) Confirmation of cell surface expression of TNF receptors by FACS. B16 murine melanoma cells, wt, TNFR1$^{-/-}$, TNFR2$^{-/-}$ MEFs were used for FACS staining with isotype (grey), TNFR1 (red line), and TNFR2 (green line) antibodies. (C) Different genotype MEFs were treated with 10 ng/ml TNF in serum-free medium for 6 h (left panel) or 1 h (right panel). Shown is the mean + SD. n = 3 (left panel) or n = 4 (right panel) both in triplicates. Paired samples t-test: *P< 0.05, **P< 0.005.

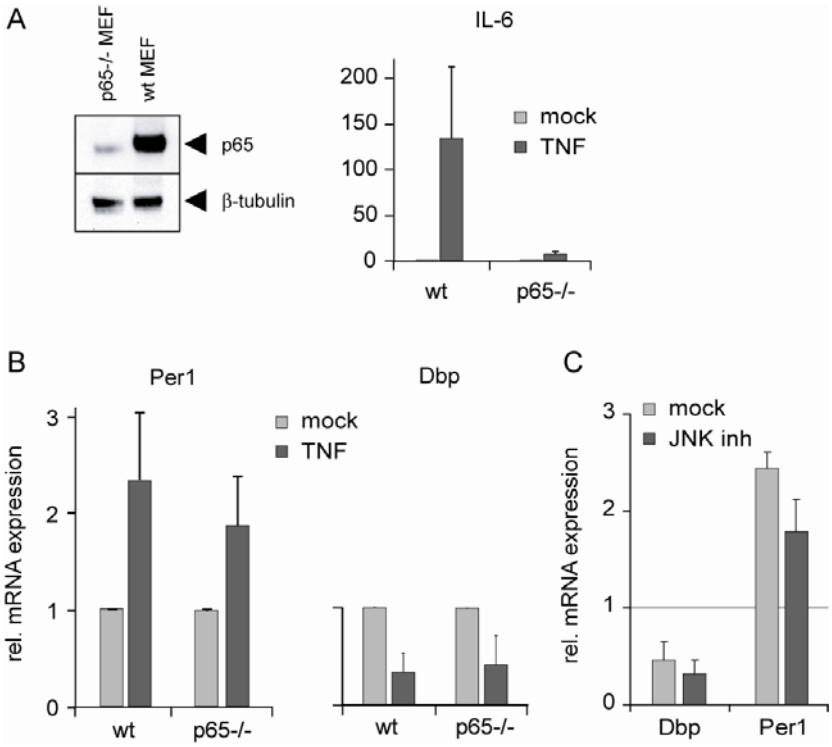



**Fig. 5: The c-jun N-terminal kinase and p65 are not involved in the early effects of TNF on clock genes.** (A) $p65^{-/-}$ MEFs do not express p65 and fail to induce IL-6 upon TNF stimulation. Left panel: wt or $p65^{-/-}$ MEFs were lysed and subjected to Western blot analysis. b-tubulin was used as a loading control. Right panel: wt or $p65^{-/-}$ MEFs were treated with 10 ng/ml TNF in serum-free medium for 4 h (Dbp panel) or 1 h (Per1 panel). Shown is the mean + SD; n = 4 in triplicates. There is no significant difference between wt and $p65^{-/-}$ MEFs. (C) A JNK inhibitor prevents neither the *Per1* peak nor the *Dbp* repression. NIH 3T3 fibroblasts were pretreated with 1μM inhibitor for 1 h and then stimulated with 10 ng/ml TNF for another hour. 1 represents the expression level in untreated cells. n = 3 in triplicates.

## *Per1* peak and *Dbp* inhibition induced by TNF are dependent on calcium ion signaling and activation of p38 MAPK

Next we assessed the role of intracellular $Ca^{2+}$ in the TNF induced clock gene response. As previously reported, we confirmed that calcium ionophores (ionomycin and PMA) induce *Per1,* but in addition we could also observe a downregulation of *Dbp* at the early phase (data not shown). To further confirm an involvement of intracellular $Ca^{2+}$ signalling in the regulation mechanism, we used the intracellular calcium chelator BAPTA-AM. The addition of BAPTA-AM effectively reduced the *Per1* peak after 1 h treatment of NIH 3T3 fibroblasts with TNF. Moreover, BAPTA-AM also effectively blocked the repression of *Dbp.* On the other hand ethylene glycol tetraacetic acid (EGTA), which chelates extracellular calcium and thereby inhibits influx of extracellular calcium, had only minor effects (Fig. 6A). Given these

results, we find TNF acting on clock gene expression by modulation of intracellular calcium. In the attempt to elucidate the implication of further TNF signalling enzymes, we tested several kinase and phoshatase inhibitors. However, blocking the protein phosphatases (PP) 1 and PP2a or the kinases phosphoinositide-3-kinase, extracellular signal regulated kinase I and II, or calcium/calmodulin dependent kinase II had no influence on clock gene regulation by TNF. Solely, the inhibition of the MAPK p38 was able to prevent the *Per1* peak (Fig. 6B). However *Dbp* expression inhibition was not affected.

Taken together, the data suggests that *Per1* induction by TNF is dependent on intracellular calcium and p38 signalling.

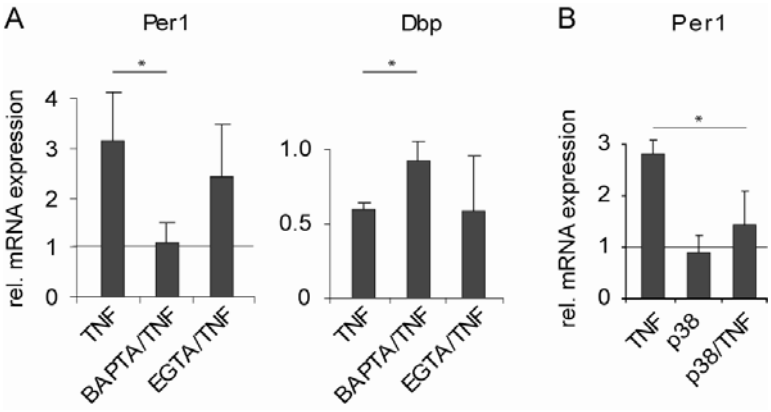



**Fig. 6: The inhibition of *Dbp* is mediated by intracellular $Ca^{2+}$ and the induction of *Per1* is mediated by intracellular $Ca^{2+}$ and p38 MAPK.** (A) Effect of the intracellular calcium chelator BAPTA-AM on *Per1* induction and *Dbp* inhibition by TNF. NIH 3T3 were treated with EGTA (1 µM) or BAPTA-AM (30 µM) 30 min before a 1 h stimulation with 10 ng/ml TNF. Expression of untreated samples corresponds to 1. Shown is the mean + SD; n = 3-5 in triplicates; (B) Effect of p38 MAPK inhibition on the *Per1* peak induced by TNF. NIH 3T3 were treated with SB203580 (10 µM) 30 min before a 1 h stimulation with 10 ng/ml TNF. Expression of untreated samples corresponds to 1. Shown is the mean + SD. n = 4 in triplicates. For A and B we used paired samples t-test: *P< 0.05.

## Chromatin remodelling defines clock gene regulations

To analyze the chromatin remodelling on the *Dbp* gene after TNF stimulation, we performed chromatin immunoprecipitation (ChIP) assays against acetylated histone H3. There is clearly less acetylated histone H3 at the *Dbp* E-box 857 corresponding to the less activity in gene transcription. Therefore, we aimed to block deacetylation by using Trichostatin A (TSA) (HDAC 2 und 3) or splitomicin (HDAC 1). We did not find any significant effects on the inhibition of *Dbp* by TNF using TSA or splitomicin. These results suggest that active deacetylation is not important for blocking gene transcription of clock genes.

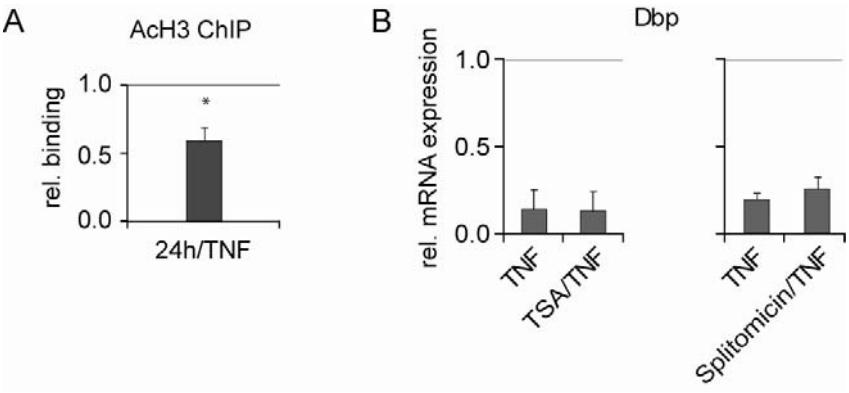



**Fig. 7: Less acetylated histone H3 at the Dbp E-box 857 corresponds to reduced expression, but active deacetylation is not involved in the inhibition of Dbp by TNF.** (A) Less acetylated histones H3 at the *Dbp* E-box 857 in cells treated with TNF assayed by ChIP. NIH 3T3 fibroblasts were synchronized by a serum shock and stimulated with 10 ng/ml TNF in serum-free medium. After 24 h, at the peak of *Dbp* gene expression, chromatin was isolated and subjected to ChIP assay. Shown is the relative binding of acetylated histones compared to unstimulated cells. n = 3 in duplicates or triplicates. (B) Trichostatin A and splitomicin have no effect on the inhibition of *Dbp* by TNF. After a 6 h or overnight pretreatment with TSA (10 ng/ml) or splitomicin (120 µM) NIH 3T3 fibroblasts were stimulated with 10 ng/ml TNF for 3 h. 1 is the expression level in mock treated cells. Shown is the mean + SD. n = 3 for TSA, n = 2 for splitomicin, both in triplicates; For A and B we used paired samples t-test: *P< 0.05.

## DISCUSSION

The biology of circadian rhythms in mammals is dependent on the appropriate function of the molecular clock within all cells of the body. The activation of the immune system parallels the deregulation of circadian functions during the acute phase of infectious and autoimmune diseases; it is therefore intriguing to speculate that this may be linked to the sickness behaviour syndrome. Recently, TNF was found to impair locomotor activity in mice and to impair E-box driven clock gene expression [92]. Fibroblasts treated for 24 h with TNF respond with a decrease of E-box mediated transcription of *Dbp*, *Tef* and *Hlf* and of *Period 1-3*. The data on the effect of TNF on fibroblasts presented here show that TNF treatment for only 1 h induces *Per1*. This effect was not associated with synchronization of the fibroblasts (data not shown), although a striking characteristic of the synchronization of cells is the induction of the clock gene *Per1* mRNA and protein. This may be a necessary event for the induction of circadian gene expression, but it is probably not sufficient as shown for epidermal growth factor which strongly induces *Per1* but still does not induce circadian gene expression [139].

Surprisingly, we found that besides *Per1,* TNF can also transiently induce other negative elements of the clock such as *Per2*, *Cry1* and *Dec1*. Because blockade of *de-novo* protein synthesis partially reverses *Dbp* inhibition, the increase in negative regulators could be partially responsible for TNF mediated impairment of *Dbp* expression. However, when assaying *Per1*, *Per2*, and *Dec1* deficient cells, we have not found direct evidence for this hypothesis. *Dec1* and *Dec2* have been reported to be regulated by several cytokines and *Dec1* is also induced by TNF in our *in-vitro* setting [140]. Even though we did not analyze the effect of *Cry*, there is indirect evidence that also *Cry* is not responsible for TNF induced downregulation of *Dbp* expression, because the heterodimerization of CRY:PER proteins should also be impaired in our *Per* deficient fibroblasts. Furthermore the induction of *Cry1* expression appears fairly late when *Dbp* is already strongly inhibited. These data strongly suggest that neither *Per1* and *Per2*, nor *Dec1* and *Cry1* are involved in *Dbp* repression. However, it still cannot be excluded that in *Per1/Per2* double knockout cells *Dec1* may lead to suppression of *Dbp* expression. Moreover, *Per1* and *Per2* may compensate for *Dec1* deletion.

TNF mediates its function by TNF receptors, TNFR1 and TNFR2. By using knockout fibroblasts, we observed that signalling via TNFR1 is sufficient to detect the early *Per1* upregulation and early as well as late *Dbp* downregulation. There are evidences that soluble murine TNF has a higher affinity to TNFR1, and that ligand binding to TNFR2 is short and

unstable [141]. We detected no cell activation by measuring IL-6 expression in TNFR1 deficient cells, still expressing TNFR2 on the surface, indeed emphasizing the higher relation of soluble TNF to TNFR1. We were surprised to find that the main TNF signalling pathways, comprising the NF-κB, JNK and caspase pathways are not responsible for clock gene regulation and that rather a "side" pathway, the $Ca^{2+}$ signalling, is mediating the E-box dependent effect of TNF. A role for calcium signalling in circadian clocks has been extensively studied in the SCN. There it has been shown that not only $Ca^{2+}$ can regulate clock gene expression but also that the circadian clock controls intracellular $Ca^{2+}$ rhythms [142-144]. $Ca^{2+}$ influx is an initial cellular event in response to glutamate stimulation by the retinohypothalamic tract in the SCN, thereby disturbing the intracellular $Ca^{2+}$ homeostasis. It has therefore been proposed to be one of the messengers conveying environmental time signals to the endogenous clock. The *Per1* gene is first induced before it is inhibited at later time points. This induction is dependent on the activation of p38 and intracellular $Ca^{2+}$. These to messengers are well described to lead to a phosphorylation of the cAMP responsive element (CRE) binding protein (CREB). In the SCN, the binding of CREB to CRE sites in the *Per1* promoter in turn induces gene expression [145-146]. Although it remains to be further investigated, we think that *Per1* expression is first induced through its CRE sites and thereafter inhibited through its E-box sequences. Here, we report the novel finding of clock gene expression regulation by $Ca^{2+}$ signalling outside of the SCN. It therefore seems that $Ca^{2+}$ plays a central role in the periphery as well. We think that analogue to the SCN, $Ca^{2+}$ could also be considered as a physiological sensor in the periphery, transmitting immunological changes to the molecular clock and thereby coordinating the special needs to specific time windows.

## MATERIAL AND METHODS

**Cell lines, cytokines and chemicals.** Murine fibroblast cells, NIH 3T3 (CRL-1658) were obtained by the American Type Culture Collection; human keratinocyte cells, HaCaT, knockout for *Dec1*, were generously provided by B. Vogelstein [147]. Embryos of wt (C57Bl/6), TNFR1$^{-/-}$, TNFR2$^{-/-}$ mice were used at day 12 of gestation (E12). The liver and heads were removed and the remaining tissue was digested with 0.25% trypsin-EDTA in DMEM for 30 min at room temperature. The dissociated cells were plated in DMEM containing 20% FCS. They were split 1:4 until they reached passage 10. Cells were used within passage 10 - 30. *p65*$^{-/-}$ and control MEFs were generously provided by M. Hottiger (University of Zürich) [148]. *mPer1*$^{-/-}$ and *mPer2*$^{-/-}$ single knockout and *mPer1*$^{-/-}$*;mPer2*$^{-/-}$ double knockout MEFs were generously provided by U. Albrecht (University of Fribourg) [134].

Recombinant murine (rm) TNF, was purchased from Peprotech (London, U.K.); BAPTA-AM, Cycloheximide, EGTA, splitomicin, actinomycin D from Sigma (ST.Louis, MO, USA); SB203580 from EMD chemicals (Darmstadt, Germany) and Trichostatin A from InvivoGen (St. Diego, USA).

**Cell culture and stimulation.** NIH 3T3 fibroblasts were grown in DMEM (Gibco, Basel, Switzerland) supplemented with 10% FBS (PAA Laboratories, Pasching, Austria) and Glutamax (Gibco). For stimulation cells were grown to confluency and then medium was replaced with serum-free DMEM/Glutamax with or without TNF. After stimulation, tissue culture plates were washed once with ice-cold PBS solution and kept at –70 °C until the extraction of whole-cell RNA.

**FACS staining.** Cells were trypsinized and resuspended in FACS buffer (PBS, 0.5% BSA, 0.02% Sodium azide) at 4°C. Antibody staining was performed with 1:100 isotype-ab (553951, BD Pharmingen), TNFR1-ab (559915, BD), TNFR2-ab (559916, BD) followed by 1:200 secondary biotinylated Goat anti-Hamster antibody (127-065-160, Jackson Immunoresearch), then with 1:200 SAV-APC (554067, BD). Staining was analyzed with FACS analyzer (Partec, Germany). Curves were smoothed for better visualization of the results.

**Western blot.** Cells were lysed in RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA 1 mM PMSF, 1% NP40, 10% glycerol, protease inhibitors) and protein concentration was measured using BCA (Pierce). 20 µg of total proteins were run with Tris-Tricine SDS-PAGE [149]. Western blotting was performed at 4°C and blot was blocked with 5% milk powder (Biorad) in TBS-T overnight. Staining was performed with 1:5000 p65

antibody (sc-372 X, Santacruz), and 1:100'000 goat anti-mouse-HRP ab (Jackson Immunoresearch), and ECLplus (Amersham) reaction. Same blots were re-stained with 1:2000 β-tubulin antibodies (Abcam).

**RNA isolation and gene expression analysis.** Whole-cell RNA from cultured cells was extracted using TRIzol (Invitrogen) or peqGOLD RNAPure (peqLab) according to the manufacturer's instructions. Subsequently, RNA was reverse-transcribed using random hexamers (Roche) and M-MuLV reverse transcriptase (Applied biosystems). The cDNA equivalent to 20 ng of total RNA was PCR-amplified in an ABI PRISM HT7900 detection system (PE-Applied Biosystems) using the TaqMan Universal PCR Master Mix (Applied Biosystems) and quantified as follows. Primers and probes for Taqman analysis were either purchased from Applied Biosystems or purchased from Microsynth, Balgach Switzerland as described in detail in SI table 1. The relative levels of each RNA were calculated by the $2^{-\Delta\Delta Ct}$ method (Ct standing for the cycle number at which the signal reaches the threshold of detection); *Gapdh* mRNA was used as a housekeeping gene. Each Ct value used for these calculations is the mean of two duplicates of the same reaction. Relative RNA levels are expressed as x-fold variations compared to untreated.

**Chromatin immunoprecipitations.** All steps were performed at 4 °C or on ice. About 1 $x10^7$ cells per condition were used as starting material. Cells were fixed for 5 min at 37 °C with 1x PBS containing 1% formaldehyde. Subsequently, cells were washed twice with ice-cold 1x PBS and scraped in Glycin buffer (125 mM Glycine; 100 mM Tris, pH 9.4; 10 mM DTT). To stop fixation, cells were kept on ice for 15 min. After a short spin of 1,5 min, 2000 g, pelleted cells were resuspended in buffer I (0.25% Triton x-100; 10 mM EDTA; 1 mM EGTA; 10 mM HEPES, pH 6.5) by vortexing. Cells were pelleted at 2000 g, 5 min and carefully resuspended in buffer II (200 mM NaCl; 1 mM EDTA; 1 mM EGTA; 10 mM HEPES, pH 6.5). After another centrifugation for 5 min, 2000 g, nuclei were carefully resuspended in 190 µl nuclei resuspension buffer at RT (165 mM NaCl; 2.2 mM EDTA; 22 mM Tris-HCl, pH 8). After addition of 10 µl 20% SDS, nuclei were submitted to 5 x 1 min sonication steps with 30 sec pauses in between using the Bioruptor™ sonication machine (Diagenode, Liège, Belgium). The fragmented cross-linked chromatin was then diluted tenfold in dilution buffer (1.1% Triton X-100; 2 mM EDTA; 150 mM NaCl; 20 mM Tris-HCl, pH 8). 100 µl chromatin was used as starting material for DNA quantification experiments and 1 µl was used as input measurement. Immunoprecipitated DNA fragments captured by protein A–agarose/salmon sperm DNA (Millipore, Billerica, MA, USA) were washed sequentially with 500ml TSE I (20 mM Tris-HCl, pH 8; 150 mM NaCl; 2 mM EDTA; 0.1% SDS; 1% TX-100), TSE II (20 mM

Tris-HCl, pH 8; 500 mM NaCl; 2 mM EDTA; 0.1% SDS; 1% TX-100), buffer III (0.25 M LiCl; 1% NP-40; 1% DOC; 1 mM EDTA; 10 mM Tris-HCl, pH 8) and TE buffer (10 mM Tris-HCl, pH 8; 1 mM EDTA) and the cross-links, including inputs, were reversed overnight at 65 °C in 20 mM Tris-HCl, pH 7.5; 150 mM NaCl; 2 mM EDTA; 1% SDS. DNA fragments were recovered using the DNA columns from the Qiagen MiniPrep kit according to the manufacturer's protocol. Two microliters of each reaction were directly used in 10 µl real-time PCR reactions using a 7900 HT machine (Applied Biosystems). The amount of DNA was quantified as described above. Each value was normalized to the corresponding input. Three independent experiments were done in duplicate or triplicate. The primers and Taqman probes used are listed in table 1. The antibody against acetylated histone H3 was purchased from Millipore.

**Nascient transcript assay.** All steps were performed on ice or at 4 °C. 2 x$10^7$ cells were scraped and washed twice with PBS, resuspended in 400 µl of HB 0.3 M sucrose (10% glycerol, 0.3 M sucrose, 60 mM KCl, 15 mM NaCl, 15 mM HEPES, pH 7.9, 0.5 mM EDTA, 0.15 mM spermine, 0.5 mM spermine, 0.5 mM PMSF, 1 mM DTT) and lysed by the addition of 400 µl HB 0.3 M sucrose containing 0.8% NP-40. After a 10 min incubation, nuclei were pelleted through a 1.2 ml cushion of HB 0.9 M sucrose by a 10 min centrifugation at 1500 g and resupended in 500 µl of nuclear suspension buffer (75 mM NaCl, 20 mM Tris-HCl, pH 7.9, 0.5 mM EDTA, 0.125 mM PMSF, 0.85 mM DTT, 50% glycerol, 100 mg/ml tRNA). Nuclei were lysed on ice for 10 min in 2400ml nuclear lysis buffer (0.3 M NaCl, 20 mM HEPES, pH 7.6, 0.2 mM EDTA, 7.5 mM MgCl2, 1 M urea, 1 mM DTT, 1 % NP-40 and 100 mg/ml tRNA). Chromatin was pelleted by 10 min at 15,000 g in a microfuge, resuspended in 500 ml of chromatin suspension buffer (50 mM Na acetate, pH 5, 50 mM NaCl, 0.5% SDS, 100 mg/ml tRNA), extracted three times with hot phenol mix (acid phenol saturated with water; 5 mM Na acetate, pH 5; 5 mM NaCl) at 65 °C. After addition of NaCl to a final concentration of 150 mM, nucleic acids were precipitated with ethanol, resuspended in 50 ml 10 mM Tris-HCl, pH 7.5, and treated with RNase-free DNase to eliminate genomic DNA. Nascent transcripts were then extracted with phenol-chloroform, precipitated and resuspended in 30 µl of 10 mM Tris-HCl, pH 7.5. 10 µg were used for reverse transcription and real-time PCR, which were performed as described above. Results were corrected for cDNA input by amplifying nascent gapdh transcripts. The primers and Taqman probes used are listed in table 1.

FOOTNOTES

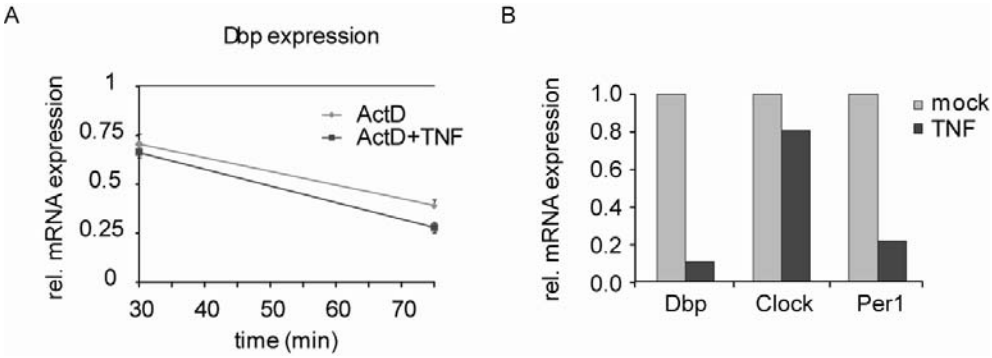
This work was supported by the Swiss National Science Foundation (Project no. 310000-109469/1 and NCCR Neural Plasticity and Repair, project 6), the Swiss Multiple Sclerosis Society, and the Gianni Rubatto Foundation.

The abbreviations used are: tumour necrosis factor, TNF; D site albumin promotor binding protein, Dbp; nuclear factor-kappaB, NF-κB; activator protein-1, AP-1; mouse embryonic fibroblasts, MEF;

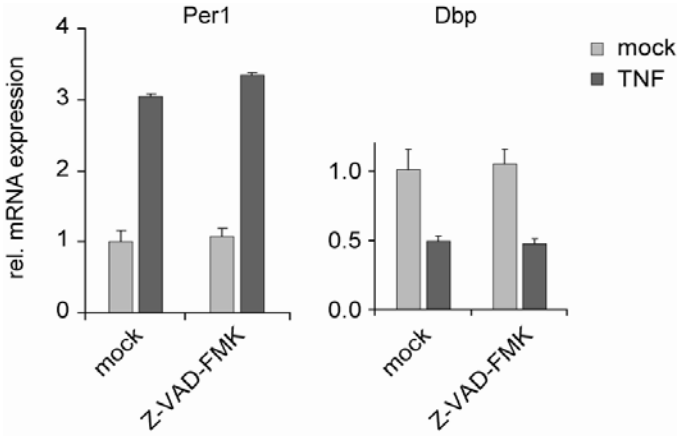
ACKOLEDGMENT

We thank Dr. J. Ripperger, and Prof. U. Albrecht for *Per* deficient MEFs.

## SUPPORTING INFORMATION




**Supplementary figure 1. The reduced levels of *Dbp* mRNA in NIH 3T3 fibroblasts is not due to an accelerated mRNA degradation.** (A) *Dbp* mRNA degradation is very rapid. Cells were treated with 1 μg/ml Act D with or without 10 ng/ml TNF and collected after 30 or 75 min for mRNA quantification. Values are relative to untreated samples collected at time point 60 min. n = 1 in triplicates. (B) Cells were stimulated with 10 ng/ml TNF overnight or left untreated. RNA was prepared for nascent transcripts analysis. n = 1 with one sample per condition.




**Supplementary figure 2. Caspases are not involved in *Per1* and *Dbp* regulations.** NIH 3T3 cells were treated with 10 ng/ml TNF with or without 50µM Z-VAD-FMK simultaneously for 1 h. Values are relative to samples without TNF stimulation. Shown is one experiment done in triplicates.

**Supplementary Table 1.** Sequences of primers and probes.

| Assay | Gene | FP | Probe | RP |
|---|---|---|---|---|
| nascent transcripts | *clock* | GGGATGGGTGGGTAATGTCA | CAGAGAGTATTGGTACACAGGACAGCGTAGCA | ACTACAGTTCTTAGGCGCACAGATG |
| | *dbp* | CTACGTTCCCGGATCCTACAC | CCCATTCCCCAAGTGGATGGTGTT | GATCCCGATATTCTCTTCTGCAA |
| | *per1* | ACCAGCCATTCCGCCTAAC | CTGCCTACTCATTGCCGAGCG | CCTAGCCTATCCACCTTCATAACC |
| ChIP | *Dbp 857* | ATGCTCACACGGTGCAGACA | CCTAGTTTCCATGTGACCCTGCGAGG | CTGCTCAGGCACATTCCTCAT |
| PCR | *tnfr1* | ACCAAGTGCCACAAAGGAAC | | CACGCACTGGAAGTGTGTCT |
| | *tnfr2* | TACCAAGGGTGGCATCTCTC | | TCCTGGGATTTCTCATCAGG |
| real time PCR | *dbp* | GCGAGAAGTGCAAAATTGGC | CGCGCGCCTGTGTCCCTTG | CGGGAGGCTCCTATAGTCTGG |
| | *clock* | TTGCTCCACGGGAATCCTT | ACACAGCTCATCCTCTCTGCTGCCTTTC | GGAGGGAAAGTGCTCTGTTGTAG |
| | *cry1* | CTGGCGTGGAAGTCATCGT | CGCATTTCACATACACTGTATGACCTGGACA | CTGTCCGCCATTGAGTTCTATG |
| | *cry2* | TGTCCCTTCCTGTGTGGAAGA | CAGTCACCCTGTGGCAGAGCCTGG | GCTCCCAGCTTGGCTTGA |
| SYBR green | *per1 1A* | TTACTCTGGAGCCGTCGAACTTGT | | AGGACGAAACAGGGAAGGTGAAGA |
| | *per1 1B* | GAACGGCCAGGTGTCGTGATTAAA | | AGGACGAAACAGGGAAGGTGAAGA |
| | | Pre-developed taqman assays (Applied biosystems) | | |
| real time PCR | *gapdh* | 4352339E | | |
| | *per1* | Mm00501813_m1 | | |
| | *per2* | Mm00478113_m1 | | |
| | *per3* | Mm00478120_m1 | | |
| | *dec1* | Mm00478593_m1 | | |
| | *dec2* | Mm00470512_m1 | | |
| | *IL-6* | Mm00446190_m1 | | |
| | *human gapdh* | 0-0702011 | | |
| | *human dbp* | Hs00609747_m1 | | |

## 4.3 Hypothalamic neurons develop an inflammatory phenotype in response to TNF

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**ABSTRACT**

Tumour necrosis factor plays a key role in numerous sleep disorders. The hypothalamus is involved in the regulation of sleep and loss of hypothalamic neurons is a hallmark of narcolepsy. In order to identify TNF mediated neuronal dysfunctions we addressed the effect of TNF on hypothalamic neurons. The data show that TNF induces the expression of interferon type I and activates their downstream signalling pathways. Furthermore, TNF activates the transcription of chemokine and adhesion molecule genes. Thus, at least in-vitro, hypothalamic neurons respond to TNF by building up a protective state aimed at recruitment of leukocytes.

**INTRODUCTION**

Tumour necrosis factor (TNF) is a pleiotropic cytokine which is involved in many processes under both physiological and pathological conditions, ranging from cell growth and differentiation, to inflammation and mediation of immune responses, to regulation of apoptosis and necrosis. TNF is produced mainly by activated macrophages, lymphocytes and natural killer cells. In the CNS, TNF is also secreted by microglia and astrocytes [30]. There is increasing evidence suggesting that TNF plays a role in the pathology of sleep disorders such as narcolepsy, sleep apnoea, as well as fatigue associated with autoimmune diseases and infections. TNF concentration was found to be higher in narcoleptic patients compared to control subjects [150]. In patients with chronic insomnia, excessive daytime sleepiness, and obstructive sleep apnoea syndrome the circadian rhythm of TNF secretion was found to be different compared to healthy volunteers. The peak of secretion shifted from night-time to daytime [151].

The hypothalamus is considered a key centre for sleep regulation. Hypothalamic neurotransmitters are involved in many regulatory processes [152]. The hypocretin / orexin system is a critical regulator of sleep/wake state, feeding behaviour and reward processes. Hypocretin -1 and -2 are exclusively synthesized by neurons in the lateral hypothalamus. Loss of these neurons results in narcolepsy and cataplexy.

In this report, we analysed the TNF-inducible gene-expression profile in murine hypothalamic neurons using DNA microarray technology. The data point to a strong focus of TNF to induce the expression of chemokine and chemokine receptor genes and of genes involved in cell migration and apoptosis.

## METHODS

**Cell culture and stimulation.** mHypoE-N1 cells were grown in DMEM (Gibco, Basel, Switzerland) supplemented with 10% FBS (PAA Laboratories, Pasching, Austria) and Glutamax (Gibco). For stimulation, cells were grown to confluency and the medium was replaced with serum-free DMEM/Glutamax with or without 10 ng/ml TNF.

**RNA extraction.** After stimulation, cells were washed with cold PBS and lysed with peqGOLD RNA pure (PeqLab, Erlangen, Germany) according to the manufacturer's instructions. Isolated RNA was thereafter DNA digested and purified with spin columns NucleoSpin RNA II (Macherey-Nagel, Düren, Germany).

**Gene expression arrays**. Quality and quantity control of each RNA sample was performed on the 2100 Bioanalyzer. Gene expression array analysis was performed in the Functional Genomics Center Zurich using the Agilent Microarray system (Agilent Technologies). Cy3/Cy5-labeled cRNA were hybridized on Agilent GE Arrays (G4846A, Agilent Technologies) according to manufacturer's protocol. The microarray analysis was performed in triplicates (3 diluent vs. 3 TNF treated). Slides were scanned using the Agilent DNA microarray scanner and the hybridization images were analysed with FeatureExtraction™ software. Genes were filtered according to their fold change and only genes exceeding a fold change of 2 up or down are reported.

**Flow cytometry staining.** After stimulation, cells detached with 1 mM EDTA in PBS and resuspended in FACS buffer (PBS, 0.5% BSA, 0.02% sodium azide) at 4 °C. Antibody staining was performed with 1:100 isotype control antibodies (553954 BD Pharmingen and 400406 Biolegend), VCAM1 antibodies (11-1061 eBioscience), FAS (554258, BD).
Acquired data were analyzed using FlowJo software.

**Protein analysis.** After stimulation, culture supernatants were collected and snap frozen in liquid nitrogen. Chemokine concentration was analyzed by Luminex technology at Cytolab, Dällikon, Switzerland.

## RESULTS AND DISCUSSION

Upon treatment of murine hypothalamic neurons (mHypoE-N1) with TNF, we found 456 genes to be upregulated and 150 genes to be downregulated more than 2 fold. As shown in figure 1, the main groups of the upregulated genes comprise chemokines, their receptors, cytokines including interferons and their target genes, and genes involved in apoptosis. The genes induced more than 2 fold are listed in Table 1.

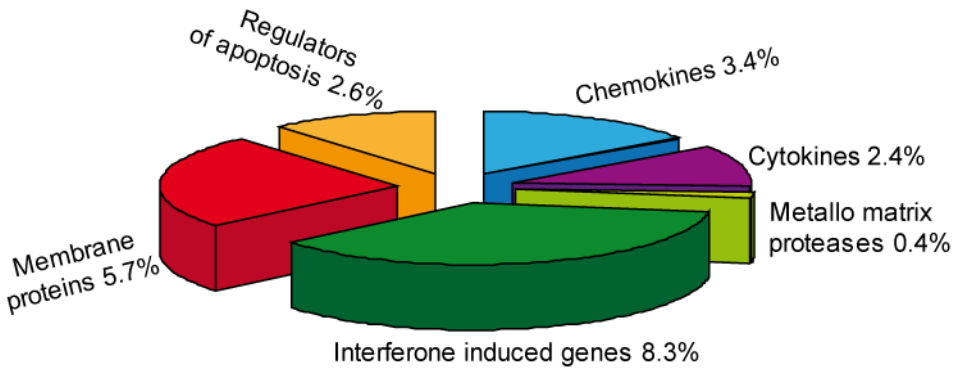


**Figure 1. .** 22.8 % of the TNF induced genes are involved in cell-cell interaction, anti-viral protection and regulation of apoptosis. The genes represented in the chart are those induced more than 2 fold.

Table 1. Genes upregulated more than 2-fold in TNF treated mHypoE-N1

| Gene name | fold induction | basal expression levels | Group description |
|---|---|---|---|
| CCL 5 / RANTES | 66.8 | M | chemokines |
| Ccl 20 / MIP-3 | 60.7 | L | chemokines |
| ICAM1 / CD54 | 38.1 | L | membrane protein |
| Cxcl 10 / IP-10 | 34.8 | H | chemokines |
| Cxcl 11 / I-TAG | 32.1 | L | chemokines |
| Ifit3 IFN-induced protein (GARG-49) | 16.1 | H | IFN related |
| Ccrl 2 | 15.8 | L | membrane protein |
| Irg1 Immune-responsive gene 1 | 13.2 | L | IFN related |
| Ifit1 1 IFI-56K /GARG-16 | 12.7 | H | IFN related |
| Cxcl 1 | 12.56 | H | chemokines |
| Gbp2 IFN-induced guanylate-binding protein 2 | 12.5 | H | IFN related |
| ICOS ligand / B7 | 11.8 | M | membrane protein |

| Gene name | fold induction | basal expression levels | Group description |
|---|---|---|---|
| FAS | 10.7 | H | apoptosis |
| TLR 2 | 10.4 | H | membrane protein |
| Iigp1 IFN-inducible G5Pase 1 | 10.2 | M | IFN related |
| MMP-13 | 9.9 | M | metalloprotease |
| Ccl 7 / MCP-3 | 9.7 | H | chemokines |
| Ifi47 IFNgamma-inducible protein 47 | 9.4 | M | IFN related |
| Ccl 11 / Eotaxin | 9.1 | L | chemokines |
| Cxcl 16 | 9.1 | M | chemokines |
| Gbp3 guanylate-binding protein 4 | 8.7 | H | IFN related |
| Mx2 IFN-induced GTP-binding protein Mx2 | 7.6 | H | IFN related |
| Naip2 Neuronal apoptosis inhibitory protein 2 | 7.6 | M | apoptosis |
| VCAM-1 / CD106 precursor | 7.5 | H | membrane protein |
| Rtp4 Receptor-transporting | 7.1 | H | IFN related |
| Ccl 17 / TARC | 6.6 | M | chemokines |
| Ccl 9 / MIP-1 | 6.5 | M | chemokines |
| Il-6 | 5.9 | L | cytokines |
| Ltbp2 | 5.7 | M | cytokines |
| Irgm2 IFN inducible GTPase 2 | 5.7 | H | IFN related |
| CD83 | 5.6 | L | membrane protein |
| Gbp1 | 5.5 | M | IFN related |
| Irf5 IFN regulatory factor 5 | 5.5 | M | IFN related |
| Cxcl 5 | 5.4 | H | chemokines |
| MMP-3 | 5.4 | M | metalloprotease |
| Birc3 Inhibitor of apoptosis protein 1 | 5.4 | H | apoptosis |
| Oas2 2'-5' oligoadenylate synthase 2 | 5.2 | M | IFN related |
| Flt1 / VEGF R1 | 5.2 | M | membrane protein |
| Mycl1 L-myc-1 proto-oncogene protein | 5.2 | M | apoptosis |
| Tnfsf18 | 5.1 | L | cytokines |
| GM-CSF | 5.1 | L | cytokines |
| Bid BH3-interacting domain death agonist | 4.5 | H | apoptosis |
| Cx3cl 1 / Fractalkine Receptor | 4.4 | H | chemokines |
| Bcl3 B-cell lymphoma 3-encoded protein homolog | 4.4 | H | apoptosis |
| Ccl8 | 4.2 | M | chemokines |

| Gene name | fold induction | basal expression levels | Group description |
|---|---|---|---|
| Ifi44 IFNgamma-inducible protein 44 | 4.2 | M | IFN related |
| ICAM -5 | 4.2 | M | membrane protein |
| IL15ra / IL-15 receptor a | 4.18 | L | membrane protein |
| Cxcl 3 / MIP-2 | 4.1 | L | chemokines |
| IL-15Ra Interleukin-15 receptor subunit alpha | 4.1 | L | apoptosis |
| Irf1 IFN regulatory factor 1 | 4 | M | IFN related |
| Trim21 SS-A / 52kd Ro | 3.9 | M | IFN related |
| IL1rl 1 /IL-1 receptor-like 1 | 3.8 | H | membrane protein |
| Ifi205 IFN-activatable protein 205-A | 3.7 | M | IFN related |
| Oas1a 2'-5' oligoadenylate synthase 1A | 3.6 | L | IFN related |
| Bmp3 / bone morphogenetic protein 3 | 3.5 | L | cytokines |
| Irgm1 Immunity-related GTPase family M protein | 3.5 | H | IFN related |
| Ifi203 IFN-activatable protein 203 | 3.5 | M | IFN related |
| Ccl 2 / MCP-1 | 3.4 | H | chemokines |
| Csf1 / M-CSF | 3.4 | H | cytokines |
| Gvin1 very large inducible GTPase 1 | 3.4 | M | IFN related |
| Il15 / IL-15 | 3.2 | M | cytokines |
| Oas1f 2'-5' oligoadenylate synthase 1F | 3.2 | M | IFN related |
| Ifnar2 IFN-a/b receptor beta chain | 3.2 | H | IFN related |
| Gpr176 G protein coupled receptor AGR9 | 3.2 | M | membrane protein |
| Ifih1 IFN-induced helicase C domain-containing protein 1 | 3.1 | M | IFN related |
| Ifi204 IFN-activatable protein 204 | 2.9 | H | IFN related |
| Irf7 IFN regulatory factor 7 | 2.7 | L | IFN related |
| Ifngr2 / IFNg receptor 2 | 2.7 | H | membrane protein |
| Oasl 2'-5' oligoadenylate synthase 1 | 2.6 | L | IFN related |
| Ifngr1 /IFNg receptor a chain | 2.6 | H | membrane protein |
| Irf9 IFN regulatory factor 9 | 2.5 | L | IFN related |
| Ifnbeta1 IFN beta | 2.5 | L | IFN related |
| Myc Myc proto-oncogene protein | 2.5 | H | apoptosis |
| Cxcl 9 / MIG | 2.4 | L | chemokines |
| Ifi35 | 2.4 | H | IFN related |
| IRF2 | 2.4 | H | IFN related |

| Gene name | fold induction | basal expression levels | Group description |
|---|---|---|---|
| Ifitm1 IFN induced transmembrane protein 1 | 2.4 | M | IFN related |
| Il13ra1 / IL-13 receptor a-1 chain | 2.4 | M | membrane protein |
| Gpm6b neuronal membrane glycoprotein M6-b | 2.4 | L | membrane protein |
| CD274 / PD-L1 | 2.4 | M | membrane protein |
| Osmr / Oncostatin-M specific receptor subunit b | 2.4 | H | membrane pro |
| Ier3 Radiation-inducible immediate-early gene IEX-1 | 2.4 | H | membrane protein |
| Trp53 Cellular tumour antigen p53 | 2.36 | H | apoptosis |
| Gdnf / glia cell line-derived neurotrophic F | 2.3 | M | cytokines |
| Ifitm6 IFN induced transmembrane protein 6 | 2.2 | M | IFN related |
| Ifitm3 IFN induced transmembrane protein 3 | 2.2 | H | IFN related |
| Ifitm7 IFN induced transmembrane protein 7 | 2.2 | H | IFN related |
| Eif2ak2 IFN-induced protein kinase | 2.2 | M | IFN related |
| Il13ra2 / IL-13 receptor a-2 chain | 2.2 | L | membrane protein |
| Wnt7b Protein Wnt-7b | 2.18 | L | apoptosis |
| Pim1 Proto-oncogene serine/threonine-protein kinase | 2.17 | M | apoptosis |
| ILrn / IL-1RA | 2.1 | L | cytokines |
| Ltb / Lymphotoxin-beta | 2.1 | L | cytokines |
| Wisp2 / connective tissue growth factor-like protein | 2.1 | M | cytokines |
| Il18rap Interleukin-18 receptor accessory protein | 2.1 | M | membrane protein |
| IL-6st / IL-6 receptor subunit b | 2.1 | H | membrane protein |
| Wisp2 / connective tissue growth factor-like protein | 2.1 | M | membrane protein |
| Il4ra / IL-4 receptor a-chain | 2.1 | M | membrane protein |
| Ifi202a IFN-activatable protein 202a | 2 | H | IFN related |
| Adora2b / Adenosin receptor A2b | 2 | M | membrane protein |

TNF treatment results in upregulation of chemokines that act either on lymphocytes and macrophages (CCL5, CCL7, CCL9, CCL17, and CCL20) or on activated T-lymphocytes (CXCL10, CXCL11, CXCL16) and neutrophils (CXCL1, CXCL5). The expression of selected chemokines as detected by microarray technology was confirmed at the protein level by Luminex techniques. As shown in Figure 2 and Table 2, neurons respond to TNF with a time-dependent and pronounced secretion of CXCL10, CCL5, CXCL1 and CCL17. Among

the genes induced by TNF we found the adhesion molecules ICAM-1 and VCAM-1 to be strongly upregulated (38.4 and 7.5 fold respectively).

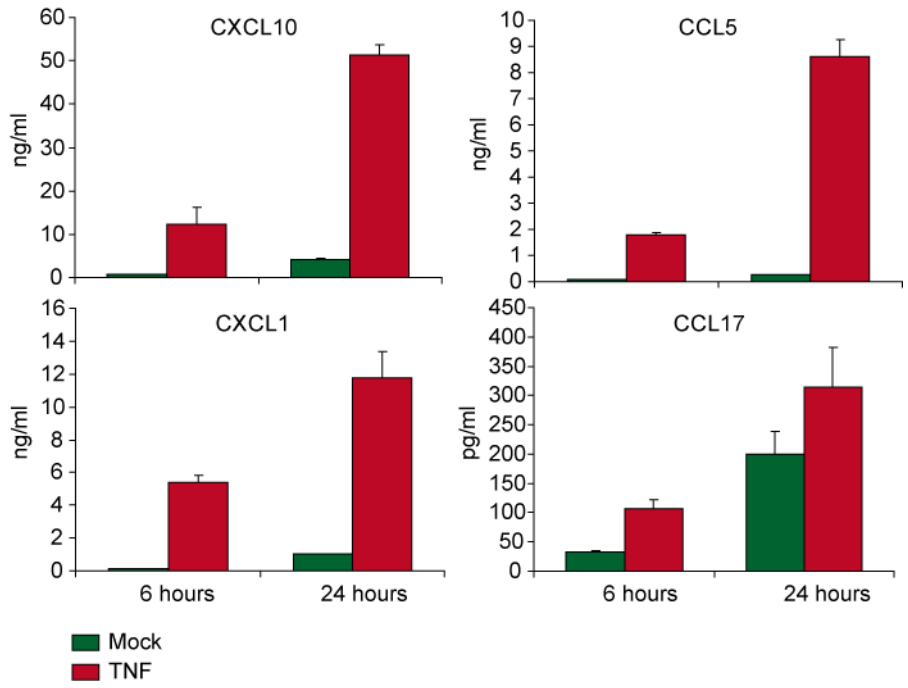


**Figure 2.** To confirm the microarray data mHypoE-N1 culture supernatants were compared after 6 and 24 h of TNF treatment by Luminex assay. Data shown means ± SD. n=3.

| Gene name | gene expression | protein concentration after TNF stimulation (ng/ml) | |
|---|---|---|---|
| | n-fold | 6 h | 24 h |
| Cxcl10 | 34.84 | 12.27 | 51.30 |
| Ccl5 | 66.86 | 1.78 | 8.63 |
| Cxcl1 | 12.56 | 5.43 | 11.79 |
| Ccl17 | 6.67 | 0.11 | 0.31 |

**Table 2.** TNF -mediated upregulation of chemokine RNA expression and protein concentration in TNF stimulated mHypoE-N1 and their respective supernatants.

The two adhesion molecules, ICAM1 (Inter-Cellular Adhesion Molecule 1) and VCAM1 (Vascular Cell Adhesion Molecule-1), which are essential in the T-cell adherence, e.g. on endothelial cells, were also among the genes strongly upregulated upon TNF treatment (38.4 and 7.5 fold respectively).

An increase of VCAM1expression on the cell membrane of TNF stimulated mHypoE-N1 became visible by FACS analysis (Fig 3A). However, ICAM1 was not detectable on the cell surface (data not shown), although, as shown by microarray, the gene expression was strongly induced. Besides the effect of TNF on chemokines, their receptors, and genes involved in cell adhesion, 2.6 % of the genes induced by TNF are involved in apoptotic signalling, including the TNF receptor family member Fas. The expression of Fas became detectable by FACS analysis, the receptor being induced on the cell surface of TNF stimulated mHypoE-N1 (Fig. 3B).

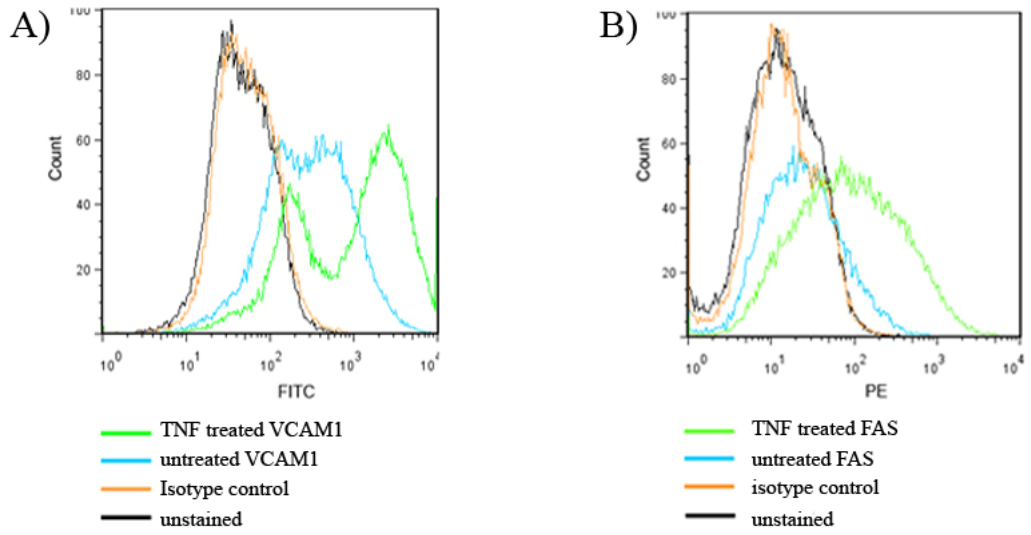


**Figure 3.** The expression of VCAM1 and Fas was found to be induced by TNF. This result were further confirmed by FACS analysis after 24 h stimulation with TNF. A) VCAM1 staining, B) FAS staining.

Taken collectively, the study presented here shows that hypothalamic neurons respond to TNF with upregulation of 450 genes. Among these 5.8 % are involved in communication with and between immune cells, such as lymphocytes, microglia / macrophages and astrocytes. It remains to be seen whether neurons of transgenic mice which express TNF in the CNS on astrocytes or oligodendrocytes also show altered expression of the genes which are found here to be modulated by TNF in mHypoE-N1 cells.

# 5. GENERAL DISCUSSION

Sickness behaviour syndrome (SBS) has evolved in the process of natural selection throughout the animal kingdom as an adaptive response to systemic infection, helping the organism to cope with infection and improving chances of survival. However, SBS accompanies numerous pathological conditions including chronic inflammation in infectious diseases or autoinflammatory diseases and autoimmune diseases. In these conditions SBS is a maladaptive behaviour which has a huge impact on the quality of life of affected individuals. The cytokines discussed to mediate these behavioural changes are TNF, IL-1 and IL-6. These cytokines coordinate the local and systemic inflammatory response and also act on the brain to cause the behavioural symptoms of sickness [9]. Although the importance of these cytokines in mediating SBS has been assessed, their functional hierarchy in the cytokine network and the mechanisms underlying the various behavioural actions of these cytokines still remain elusive.

In this thesis, a mouse model for sickness behaviour was established using CD40 ligation.

Anti-CD40 antibodies bind to CD40 on innate immune cells mimicking the exaggerated CD40 activation induced by CD40L in autoimmune diseases. Excessive signalling through CD40-CD40L interaction is critically involved in the development of numerous animal models of autoimmune diseases, including multiple sclerosis, rheumatoid arthritis, and systemic lupus erythematosus [25]. Thus, our mouse model is likely to approach the situation of SBS in patients with autoimmune diseases.

In this regard it is interesting that agonistic anti-CD40 has also therapeutic potential in the treatment of solid tumours and lymphomas, its applicability is however limited due to the occurrence of side effects such as fatigue, anorexia and headache [99].

Locomotor activity is one of the main circadian outputs and it is easily measured in rodents. Its decrease during pathological conditions belongs to the characteristics of sickness behaviour. Therefore, we monitored locomotor activity of individual mice in all our in vivo experiments. The nocturnal locomotor activity of mice treated with CD40 mAb significantly reduced the effect, returning to normal seven days after injection. The reduction in activity was paralleled by an increase in the number of sleep episodes longer than 5 minutes, indicating that as a sign for fatigue, the mice are most likely sleeping. During the light period, when mice usually rest, no increase in locomotor activity was observed, meaning that the distribution of activity remained within the normal circadian pattern.

In order to understand the individual role of IL-6, TNF and IL-1 and their functional hierarchy within the cytokine network in inducing SBS, we addressed the effect of CD40 mAb

administration in IL-6$^{-/-}$, TNFR1$^{-/-}$, IL-1R1$^{-/-}$, and MyD88$^{-/-}$ mice. Interestingly, all knockout strains analysed differed in their behavioural response to CD40 mAb treatment. Concerning nocturnal running wheel and infrared activity, IL6$^{-/-}$ mice responded similarly to wild type (see above), IL-1R1$^{-/-}$ showed a moderate response, whereas TNFR1$^{-/-}$ and MyD88$^{-/-}$ mice were completely protected from CD40 mAb mediated SBS.

All three cytokines investigated here and especially IL-1 and TNF share numerous biological activities and induce the synthesis and release of each other. Moreover, IL-1 and TNF often exert additive or synergistic effects [42]. The complete unresponsiveness to development of CD40 mAb induced SBS as observed in TNFR1$^{-/-}$, and the moderate response of IL-1R1$^{-/-}$ may point to the role of TNF to induce IL-1, which itself in a downstream step may amplify the action of TNF. This hypothesis was further validated by the observation that TNF injections mimic the effect of CD40 mAb and even restore the resistance seen in MyD88$^{-/-}$ mice. CD40 mAb challenged MyD88$^{-/-}$ mice had reduced levels of TNF in serum compared to wild type, suggesting that MyD88-mediated signalling events may be required for production of TNF in quantities necessary for SBS induction. This signal, though, is not transduced by IL-1 because IL-1R1$^{-/-}$ were still responsive to CD40 mAb. The intracellular adaptor protein MyD88 is used, not only by IL1R and IL18R, but also by all Toll like receptors (TLRs) (except TLR3) and interferon-regulated factors [102]. This suggests that a TLR ligand or IRF-mediated signalling is required to induce TNF synthesis in MyD88 deficient mice. Though, the nature and the source of this signal remain elusive.

Another important feature of SBS is weight loss which can arise from anorexia or cachexia, a highly complex metabolic disorder involving features of anorexia, anaemia, lipolysis and insulin resistance [153]. As true for locomotor activity, we observed difference in the degree of weight loss among the genotypes analysed. Compared to wild type, also IL-6$^{-/-}$ showed significant weight loss, excluding IL-6 as a possible mediator. This is surprising given the vast literature on the crucial role of IL-6 in the acute phase response and liver pathologies [154]. While weight loss in IL-1R1$^{-/-}$ was moderate, it was minimal in TNFR1$^{-/-}$. Both TNF and IL-1 have been well established to mediate progressive weight loss. TNF is considered to be the most relevant cytokine in the development of cachexia seen in chronic illnesses. Intraperitoneal injection of IL-1 induces a reduction in total caloric intake in rats, suggesting that IL-1 may also contribute to anorexia. IL-1 might activate CNS structures involved in food intake and energy homeostasis via its action on the vagus nerve [155]. Since MyD88$^{-/-}$ mice were completely protected from CD40 mAb-induced weight loss, MyD88 though is not involved in CD40 signalling. In summary, since MyD88$^{-/-}$ mice have reduced levels of TNF in

serum compared to wild type, and IL-1R1$^{-/-}$ are partially resistant, we conclude that IL-1 and TNF have additive effects in SBS. The drastic effect observed in TNFR1$^{-/-}$ may indicate that TNF is a stronger inducer of weight loss and that IL-1 potentiates its effect.

The physiological and behavioural changes that are characteristic for sickness are controlled by the central nervous system. Cytokines, including TNF and IL-1, can be synthesized and released directly within the CNS, where they are produced mainly by microglia, astrocytes and neurons [156]. It has been difficult to identify TNFR and IL-1R1 expression in the brain of healthy rodents, probably reflecting low density of receptor expression and poor sensitivity of the techniques used. Nevertheless, TNFR1 expression has been reported in multiple brain regions, including the choroid plexus, the cerebellum, the cortex and the hippocampus [157]. In contrast, TNFR2 expression was not detectable within the brain [158]. IL-1R1 expression was detected in the hippocampus, amygdala and mediodorsal thalamic nucleus. In the paraventricular hypothalamic nucleus the signal of IL-1R1 mRNA was found only on blood vessels [159-160]. Therefore, the next challenge in clarifying the mechanism of cytokine induced SBS is to identify which cell type needs to express cytokine receptors and whether these are required in the periphery or in the CNS.

In order to assess whether the expression of the receptor on immune cells invading the CNS is sufficient to induce SBS or whether the receptor has to be expressed within the CNS, we will generate bone marrow chimeras using bone marrow of TNFRI$^{-/-}$ or wt for engraftment into wt and TNFRI$^{-/-}$ mice respectively and analyse the effect of anti-CD40 treatment in these mice.

As mentioned above, in the CNS cytokines are synthesized mainly by microglia and astrocytes. However, SBS is the result of altered neuronal outputs. Therefore, it would be particularly interesting to investigate the role of TNFR1 expressed in neurons. By crossing mice expressing the Cre recombinase under the promoter of the neural stem cell specific gene *Nestin* with floxed TNFR1 mice, it is possible to knockout the TNFR1 only in CNS derived cells. Moreover, investigating the distribution of cytokine expression in the different brain regions upon CD40 mAb in wild type and the different knockout mice used by immunohistochemistry, particularly in the SCN, would further contribute to understanding where cytokine expression is essential in order to induce sickness behaviour.

The impact of circadian timing on our health has gained increasing attention in the last decade, and circadian dysfunction is thought to contribute to the incidence of a wide range of diseases, including sleep disorders, cancer, depression and inflammation. Though, a link between core clock proteins with inflammatory pathways has only recently begun to emerge. Evidence from both in vitro and in vivo studies suggests the pro-inflammatory cytokine TNF

can modulate the expression of clock genes *Period*, *Rev-Erbα* and of the clock-controlled genes *Dbp*, *Hlf* and *Tef*, both in fibroblasts *in vitro* and in the liver [92]. Here we showed CD40 mAb treatment also leads to suppression of *Period* and *Rev-Erbα* genes and of *Dbp*, *Hlf* and *Tef*. As expected, the reduced expression of *Rev-Erbα* was parallel by upregulation of *Bmal1*. *Rev-Erbα* inhibits *Bmal1* transcription by binding to RORE DNA sequences in its promoter [65]. Noteworthy, as true for CD40 mAb-mediated impairment of locomotor activity with increased rest periods, also the CD40 ligation-induced dysregulation of clock gene expression depends on TNFR1 and partially on IL-1R1, but not on IL-6. As shown by luciferase reporter assays, TNF impairs transcription of E-box containing constructs [92]. This finding was further validated here by BMAL1 chromatin immunoprecipitation, showing impaired BMAL1 binding to E-boxes in the respective *Dbp* and *Rev-Erbα* promoters at ZT9 in CD40 mAb treated animals compared to control. This effect was seen only in nuclear liver extracts obtained from CD40 mAb treated wt, but not when derived from $TNFR1^{-/-}$ mice exposed to CD40 mAb.

A series of studies indicate that activation of CCGs by CLOCK:BMAL1 is coupled to circadian changes in histone modifications at their promoter [103]. Indeed, we found that circadian transcription of *Dbp* and *Rev-Erbα* is accompanied by acetylation of Histones H3 at lysine 9. CD40 treatment lowered only the levels of AcH3K9 at the E-boxes of *Dbp* and *Rev-Erbα* in liver samples from wt mice, but not from $TNFR1^{-/-}$ mice. Thus transcriptional clock gene deactivation by CD40 as judged by acetylated H3 is dependent on TNF signalling. The transcription factor CLOCK has intrinsic histone acetyl transferase (HAT) activity. Using mouse embryonic fibroblasts derived from homozygous *Clock* mutant, it was shown that the HAT function of CLOCK is essential for the circadian control of CCGs [161]. Acetylation of proteins has been reported to be an essential regulatory mechanism, stimulating or inhibiting transcription. The dimerization partner of CLOCK, BMAL1 is acetylated by CLOCK with a diurnal rhythm in the liver. The timing of BMAL1 acetylation parallels the down regulation of circadian transcription of clock-controlled genes. Recently, the histone deacetylase sirtuin 1 (SIRT1) was identified as a crucial modulator of the circadian clock machinery by counteracting the HAT activity of CLOCK [162]. SIRT1 plays a crucial role in metabolism and survival by deacetylating several proteins and regulating gene expression through histone deacetylation. An interesting hallmark of SIRT1 is that its enzymatic activity is $NAD^+$-dependent. As the $NAD^+$/NADH ratio is a direct measure of the energy status of a cell, the $NAD^+$ dependence of SIRT1 directly links cellular energy metabolism to gene transcritpion [163]. The reduced form of both redox cofactor, NAD(H) and NADP(H) have been shown to

strongly enhance DNA-binding of the CLOCK:BMAL1 complex, whereas the oxidized form inhibits binding [164].

In this scenario it can be envisaged that an immune response may also have effects on the circadian clock. NADPH oxidase enzymes are present in most tissues and catalyze the reduction of molecular oxygen to superoxide anions by the transfer of electrons from NAD(P)H. These reactive oxygen species (ROS) contribute to clearance of pathogens in innate immunity and function as signalling molecules in various cellular processes, including apoptosis and necrotic cell death. TNF is a potent inducer of ROS production. Recently, the mechanism, through which TNF activates the NADPH oxidases NOX1 and NOX2, has been revealed. The riboflavin kinase (RFK) was identified as a TNFR1-binding protein that couples TNFR1 to NADPH oxidase. RFK binds to both the TNFR1-death domain and to $p22^{phox}$, the common subunit of NADPH oxidase isoforms NOX1 and NOX2 [165]. TNF, through activation of RFK, enhances the incorporation of the rate-limiting cofactor FAD in NADPH oxidase (Figure 5). In an *in vitro* experiment, we will investigate whether TNF-induced reduction of E-box-bearing clock genes can be reversed by inhibiting the production of ROS with antioxidants, such as N-acetyl-cystein.

In conclusion, it seems reasonable that cytokines by altering the metabolic state of cells lead to clock gene dysregulation. This in turn may lead to lowering the metabolic rate in order conserve energy by turning off appetite to avoid the urge to search for food, and by causing fatigue encouraging the animals to remain in a save place minimizing energy expenditure.

We have shown that TNF:TNFR1 interactions in CD40 mAb treated mice lead to increased rest periods and altered clock gene responses. The important question is whether these parallel outcomes are indeed linked to each other. We found significant correlations between the magnitude of CD40 mAb-triggered impairment of locomotor activity and dysregulation of clock genes in the different knockout mice studied. Mice with the most dramatic CD40 mAb-reduction in locomotor activity also showed the most pronounced decrease of *Dbp* expression. Expression of *Rev-Erbα* showed a trend in the same direction, whereas *Bmal1* expression tended in the opposite direction when correlated to reduction in locomotor activity, though both did not reach significance. Noteworthy is the intermediate expression of clock genes shown by $IL1R1^{-/-}$ and $TNFR1^{+/-}$, which is reflected in their moderate loss of activity.

We could observe an association between sickness behaviour and deregulated clock gene expression, but at this point we do not know if the impairment of the circadian clock is the cause or barely the consequence of altered physiology which accompanies the sickness behaviour syndrome. This is a challenging issue to address. Mice lacking the core clock

component *Bmal1* have completely disturbed circadian rhythms and are in this regard suitable to assess whether disturbance of the circadian clock per se has an impact on the function of the immune system. Bmal1$^{-/-}$ mice, however, because of the involvement of the core clock proteins in multiple physiological and metabolic processes, have severely compromised physiology and already at the age of 16 weeks show signs of aging, such as weight loss, loss of muscle tone accompanied by reduced locomotor activity and osteoporosis [86]. Being aware of these difficulties, we will assess cytokines in the serum of naïve and CD40 mAb challenged *Bmal1* knockout mice younger that ten weeks.

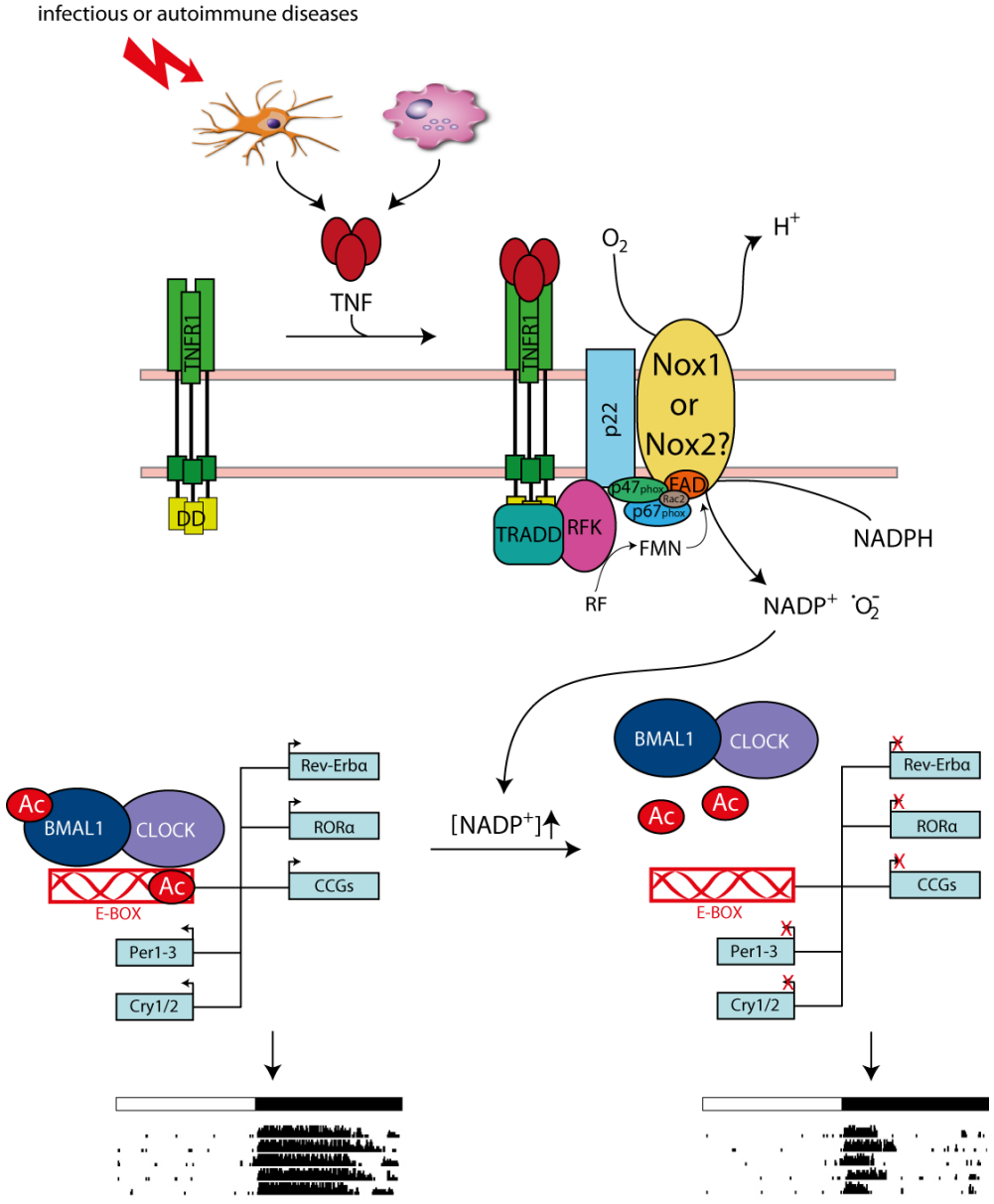


Figure 5. A schematic model illustrating a hypothetical link between TNF signalling and the circadian clock (Inspired by [164] and [165]).

The classical view of cytokine mediated sickness behaviour suggests that a peripheral infection ultimately leads to cytokines release in the brain, where they act on the different neuronal centres thereby affecting behaviour. Here, an additional mechanism emerges. Cytokines may act on peripheral organs and dampen the amplitude of their circadian clocks, and thus modifying their metabolic activity. These may in turn cause alterations in the clock outputs modifying behaviour and physiology, leading to the appearance of sickness behaviour syndrome.

# 6. REFERENCES


1. Dantzer, R., *Cytokine-induced sickness behaviour: a neuroimmune response to activation of innate immunity.* Eur J Pharmacol, 2004. **500**(1-3): p. 399-411.
2. Bernheim, H.A. and M.J. Kluger, *Fever and antipyresis in the lizard Dipsosaurus dorsalis.* Am J Physiol, 1976. **231**(1): p. 198-203.
3. Kluger, M.J. and L.K. Vaughn, *Fever and survival in rabbits infected with Pasteurella multocida.* J Physiol, 1978. **282**: p. 243-51.
4. Murray, M.J. and A.B. Murray, *Anorexia of infection as a mechanism of host defense.* Am J Clin Nutr, 1979. **32**(3): p. 593-6.
5. van Langenberg, D.R. and P.R. Gibson, *Systematic review: fatigue in inflammatory bowel disease.* Aliment Pharmacol Ther, 2010.
6. Heesen, C., et al., *Fatigue in multiple sclerosis: an example of cytokine mediated sickness behaviour?* J Neurol Neurosurg Psychiatry, 2006. **77**(1): p. 34-9.
7. Burgos, P.I., et al., *Disease activity and damage are not associated with increased levels of fatigue in systemic lupus erythematosus patients from a multiethnic cohort: LXVII.* Arthritis Rheum, 2009. **61**(9): p. 1179-86.
8. van Hoogmoed, D., et al., *Physical and psychosocial correlates of severe fatigue in rheumatoid arthritis.* Rheumatology (Oxford).
9. Dantzer, R., et al., *From inflammation to sickness and depression: when the immune system subjugates the brain.* Nat Rev Neurosci, 2008. **9**(1): p. 46-56.
10. Holmes, J.E. and N.E. Miller, *Effects of Bacterial Endotoxin on Water Intake, Food Intake, and Body Temperature in the Albino Rat.* J Exp Med, 1963. **118**: p. 649-58.
11. Tizard, I., *Sickness behavior, its mechanisms and significance.* Anim Health Res Rev, 2008. **9**(1): p. 87-99.
12. Konsman, J.P., P. Parnet, and R. Dantzer, *Cytokine-induced sickness behaviour: mechanisms and implications.* Trends Neurosci, 2002. **25**(3): p. 154-9.
13. Klune, J.R., et al., *HMGB1: endogenous danger signaling.* Mol Med, 2008. **14**(7-8): p. 476-84.
14. O'Shea, J.J., et al., *Key molecules involved in receptor-mediated lymphocyte activation.* Curr Protoc Immunol, 2001. **Chapter 11**: p. Unit 11 9A.
15. Conti, B., et al., *Cytokines and fever.* Front Biosci, 2004. **9**: p. 1433-49.
16. Akira, S., S. Uematsu, and O. Takeuchi, *Pathogen recognition and innate immunity.* Cell, 2006. **124**(4): p. 783-801.

17. Dinarello, C.A., *Infection, fever, and exogenous and endogenous pyrogens: some concepts have changed.* J Endotoxin Res, 2004. **10**(4): p. 201-22.
18. Akira, S., K. Takeda, and T. Kaisho, *Toll-like receptors: critical proteins linking innate and acquired immunity.* Nat Immunol, 2001. **2**(8): p. 675-80.
19. Gatti, S. and T. Bartfai, *Induction of tumor necrosis factor-alpha mRNA in the brain after peripheral endotoxin treatment: comparison with interleukin-1 family and interleukin-6.* Brain Res, 1993. **624**(1-2): p. 291-4.
20. Laye, S., et al., *Peripheral administration of lipopolysaccharide induces the expression of cytokine transcripts in the brain and pituitary of mice.* Brain Res Mol Brain Res, 1994. **27**(1): p. 157-62.
21. Bluthe, R.M., et al., *Role of IL-6 in cytokine-induced sickness behavior: a study with IL-6 deficient mice.* Physiol Behav, 2000. **70**(3-4): p. 367-73.
22. Cunningham, C., et al., *The sickness behaviour and CNS inflammatory mediator profile induced by systemic challenge of mice with synthetic double-stranded RNA (poly I:C).* Brain Behav Immun, 2007. **21**(4): p. 490-502.
23. Danese, S., M. Sans, and C. Fiocchi, *The CD40/CD40L costimulatory pathway in inflammatory bowel disease.* Gut, 2004. **53**(7): p. 1035-43.
24. Elgueta, R., et al., *Molecular mechanism and function of CD40/CD40L engagement in the immune system.* Immunol Rev, 2009. **229**(1): p. 152-72.
25. Koshy, M., D. Berger, and M.K. Crow, *Increased expression of CD40 ligand on systemic lupus erythematosus lymphocytes.* J Clin Invest, 1996. **98**(3): p. 826-37.
26. Durie, F.H., et al., *Prevention of collagen-induced arthritis with an antibody to gp39, the ligand for CD40.* science, 1993. **261**(5126): p. 1328-30.
27. Oliff, A., et al., *Tumors secreting human TNF/cachectin induce cachexia in mice.* Cell, 1987. **50**(4): p. 555-63.
28. Clark, I.A., *How TNF was recognized as a key mechanism of disease.* Cytokine Growth Factor Rev, 2007. **18**(3-4): p. 335-43.
29. Chen, G. and D.V. Goeddel, *TNF-R1 signaling: a beautiful pathway.* science, 2002. **296**(5573): p. 1634-5.
30. Bradley, J.R., *TNF-mediated inflammatory disease.* J Pathol, 2008. **214**(2): p. 149-60.
31. McCoy, M.K. and M.G. Tansey, *TNF signaling inhibition in the CNS: implications for normal brain function and neurodegenerative disease.* J Neuroinflammation, 2008. **5**: p. 45.

32. Hehlgans, T. and K. Pfeffer, *The intriguing biology of the tumour necrosis factor/tumour necrosis factor receptor superfamily: players, rules and the games.* Immunology, 2005. **115**(1): p. 1-20.
33. Moreland, L.W., et al., *Effect of etanercept on fatigue in patients with recent or established rheumatoid arthritis.* Arthritis Rheum, 2006. **55**(2): p. 287-93.
34. Tyring, S., et al., *Etanercept and clinical outcomes, fatigue, and depression in psoriasis: double-blind placebo-controlled randomised phase III trial.* Lancet, 2006. **367**(9504): p. 29-35.
35. Bluthe, R.M., et al., *Role of interleukin-1beta and tumour necrosis factor-alpha in lipopolysaccharide-induced sickness behaviour: a study with interleukin-1 type I receptor-deficient mice.* Eur J Neurosci, 2000. **12**(12): p. 4447-56.
36. Jiang, Y., et al., *Inhibition of peripheral TNF can block the malaise associated with CNS inflammatory diseases.* Neurobiol Dis, 2008. **32**(1): p. 125-32.
37. Rothwell, N.J. and G.N. Luheshi, *Interleukin 1 in the brain: biology, pathology and therapeutic target.* Trends Neurosci, 2000. **23**(12): p. 618-25.
38. Dinarello, C.A., *Interleukin-1beta and the autoinflammatory diseases.* N Engl J Med, 2009. **360**(23): p. 2467-70.
39. Dinarello, C.A., *Immunological and inflammatory functions of the interleukin-1 family.* Annu Rev Immunol, 2009. **27**: p. 519-50.
40. Arend, W.P., G. Palmer, and C. Gabay, *IL-1, IL-18, and IL-33 families of cytokines.* Immunol Rev, 2008. **223**: p. 20-38.
41. Goshen, I. and R. Yirmiya, *Interleukin-1 (IL-1): a central regulator of stress responses.* Front Neuroendocrinol, 2009. **30**(1): p. 30-45.
42. Bluthe, R.M., et al., *Lipopolysaccharide induces sickness behaviour in rats by a vagal mediated mechanism.* C R Acad Sci III, 1994. **317**(6): p. 499-503.
43. Fonseca, J.E., et al., *Interleukin-6 as a key player in systemic inflammation and joint destruction.* Autoimmun Rev, 2009. **8**(7): p. 538-42.
44. Ishihara, K. and T. Hirano, *IL-6 in autoimmune disease and chronic inflammatory proliferative disease.* Cytokine Growth Factor Rev, 2002. **13**(4-5): p. 357-68.
45. Heinrich, P.C., et al., *Principles of interleukin (IL)-6-type cytokine signalling and its regulation.* Biochem J, 2003. **374**(Pt 1): p. 1-20.
46. de Mairan, J.J.O., in Histoire de l'Académie Royale des Sciences, 1792: p. pp. 35-36.
47. Richter, C.P., *Sleep and activity: their relation to the 24-hour clock.* Res Publ Assoc Res Nerv Ment Dis, 1967. **45**: p. 8-29.

48. Takahashi, J.S., et al., *The genetics of mammalian circadian order and disorder: implications for physiology and disease.* Nat Rev Genet, 2008. **9**(10): p. 764-75.
49. Antle, M.C. and R. Silver, *Orchestrating time: arrangements of the brain circadian clock.* Trends Neurosci, 2005. **28**(3): p. 145-51.
50. Panda, S., et al., *Melanopsin is required for non-image-forming photic responses in blind mice.* science, 2003. **301**(5632): p. 525-7.
51. Hannibal, J., *Neurotransmitters of the retino-hypothalamic tract.* Cell Tissue Res, 2002. **309**(1): p. 73-88.
52. Abrahamson, E.E. and R.Y. Moore, *Suprachiasmatic nucleus in the mouse: retinal innervation, intrinsic organization and efferent projections.* Brain Res, 2001. **916**(1-2): p. 172-91.
53. Ralph, M.R., et al., *Transplanted suprachiasmatic nucleus determines circadian period.* science, 1990. **247**(4945): p. 975-8.
54. Ralph, M.R. and M. Menaker, *A mutation of the circadian system in golden hamsters.* science, 1988. **241**(4870): p. 1225-7.
55. Silver, R., et al., *A diffusible coupling signal from the transplanted suprachiasmatic nucleus controlling circadian locomotor rhythms.* Nature, 1996. **382**(6594): p. 810-3.
56. Kramer, A., et al., *Regulation of daily locomotor activity and sleep by hypothalamic EGF receptor signaling.* science, 2001. **294**(5551): p. 2511-5.
57. Cheng, M.Y., et al., *Prokineticin 2 transmits the behavioural circadian rhythm of the suprachiasmatic nucleus.* Nature, 2002. **417**(6887): p. 405-10.
58. Kraves, S. and C.J. Weitz, *A role for cardiotrophin-like cytokine in the circadian control of mammalian locomotor activity.* Nat Neurosci, 2006. **9**(2): p. 212-9.
59. Meyer-Bernstein, E.L., et al., *Effects of suprachiasmatic transplants on circadian rhythms of neuroendocrine function in golden hamsters.* Endocrinology, 1999. **140**(1): p. 207-18.
60. de la Iglesia, H.O., J. Meyer, and W.J. Schwartz, *Lateralization of circadian pacemaker output: Activation of left- and right-sided luteinizing hormone-releasing hormone neurons involves a neural rather than a humoral pathway.* J Neurosci, 2003. **23**(19): p. 7412-4.
61. Saper, C.B., et al., *The hypothalamic integrator for circadian rhythms.* Trends Neurosci, 2005. **28**(3): p. 152-7.

62. Damiola, F., et al., *Restricted feeding uncouples circadian oscillators in peripheral tissues from the central pacemaker in the suprachiasmatic nucleus.* Genes Dev, 2000. **14**(23): p. 2950-61.
63. Le Minh, N., et al., *Glucocorticoid hormones inhibit food-induced phase-shifting of peripheral circadian oscillators.* EMBO J, 2001. **20**(24): p. 7128-36.
64. Reppert, S.M. and D.R. Weaver, *Coordination of circadian timing in mammals*, in *Nature*. 2002. p. 935-41.
65. Hastings, M.H., A.B. Reddy, and E.S. Maywood, *A clockwork web: circadian timing in brain and periphery, in health and disease.* Nat Rev Neurosci, 2003. **4**(8): p. 649-61.
66. Grimaldi, B., et al., *Chromatin remodeling, metabolism and circadian clocks: the interplay of CLOCK and SIRT1.* Int J Biochem Cell Biol, 2009. **41**(1): p. 81-6.
67. Lee, C., et al., *Posttranslational mechanisms regulate the mammalian circadian clock.* Cell, 2001. **107**(7): p. 855-67.
68. Cardone, L., et al., *Circadian clock control by SUMOylation of BMAL1.* science, 2005. **309**(5739): p. 1390-4.
69. Etchegaray, J.P., et al., *Rhythmic histone acetylation underlies transcription in the mammalian circadian clock.* Nature, 2003. **421**(6919): p. 177-82.
70. Brown, S.A., et al., *PERIOD1-associated proteins modulate the negative limb of the mammalian circadian oscillator.* science, 2005. **308**(5722): p. 693-6.
71. Lowrey, P.L. and J.S. Takahashi, *Mammalian circadian biology: elucidating genome-wide levels of temporal organization.* Annu Rev Genomics Hum Genet, 2004. **5**: p. 407-41.
72. Ko, C.H. and J.S. Takahashi, *Molecular components of the mammalian circadian clock.* Hum Mol Genet, 2006. **15 Spec No 2**: p. R271-7.
73. Falvey, E., L. Marcacci, and U. Schibler, *DNA-binding specificity of PAR and C/EBP leucine zipper proteins: a single amino acid substitution in the C/EBP DNA-binding domain confers PAR-like specificity to C/EBP.* Biol Chem, 1996. **377**(12): p. 797-809.
74. Lopez-Molina, L., et al., *The DBP gene is expressed according to a circadian rhythm in the suprachiasmatic nucleus and influences circadian behavior.* EMBO J, 1997. **16**(22): p. 6762-71.
75. Franken, P., et al., *The transcription factor DBP affects circadian sleep consolidation and rhythmic EEG activity.* J Neurosci, 2000. **20**(2): p. 617-25.

76. Matsuo, T., et al., *Control mechanism of the circadian clock for timing of cell division in vivo.* science, 2003. **302**(5643): p. 255-9.
77. Zhu, Y., et al., *Period3 structural variation: a circadian biomarker associated with breast cancer in young women.* Cancer Epidemiol Biomarkers Prev, 2005. **14**(1): p. 268-70.
78. Filipski, E., et al., *Host circadian clock as a control point in tumor progression.* J Natl Cancer Inst, 2002. **94**(9): p. 690-7.
79. Filipski, E., et al., *Effects of chronic jet lag on tumor progression in mice.* Cancer Res, 2004. **64**(21): p. 7879-85.
80. Hansen, J., *Increased breast cancer risk among women who work predominantly at night.* Epidemiology, 2001. **12**(1): p. 74-7.
81. Jones, C.R., et al., *Familial advanced sleep-phase syndrome: A short-period circadian rhythm variant in humans.* Nat Med, 1999. **5**(9): p. 1062-5.
82. Toh, K.L., et al., *An hPer2 phosphorylation site mutation in familial advanced sleep phase syndrome.* science, 2001. **291**(5506): p. 1040-3.
83. Xu, Y., et al., *Functional consequences of a CKIdelta mutation causing familial advanced sleep phase syndrome.* Nature, 2005. **434**(7033): p. 640-4.
84. McClung, C.A., *Circadian genes, rhythms and the biology of mood disorders.* Pharmacol Ther, 2007. **114**(2): p. 222-32.
85. Turek, F.W., et al., *Obesity and metabolic syndrome in circadian Clock mutant mice.* science, 2005. **308**(5724): p. 1043-5.
86. Kondratov, R.V., et al., *Early aging and age-related pathologies in mice deficient in BMAL1, the core componentof the circadian clock.* Genes Dev, 2006. **20**(14): p. 1868-73.
87. Marcheva, B., et al., *Disruption of the clock components CLOCK and BMAL1 leads to hypoinsulinaemia and diabetes.* Nature, 2010.
88. Takahashi, J.S., K. Shimomura, and V. Kumar, *Searching for genes underlying behavior: lessons from circadian rhythms.* science, 2008. **322**(5903): p. 909-12.
89. Hayashi, M., S. Shimba, and M. Tezuka, *Characterization of the molecular clock in mouse peritoneal macrophages.* Biol Pharm Bull, 2007. **30**(4): p. 621-6.
90. Keller, M., et al., *A circadian clock in macrophages controls inflammatory immune responses.* Proc Natl Acad Sci U S A, 2009. **106**(50): p. 21407-12.
91. Arjona, A. and D.K. Sarkar, *Evidence supporting a circadian control of natural killer cell function.* Brain Behav Immun, 2006. **20**(5): p. 469-76.

92. Cavadini, G., et al., *TNF-alpha suppresses the expression of clock genes by interfering with E-box-mediated transcription.* Proc Natl Acad Sci U S A, 2007. **104**(31): p. 12843-8.
93. Okada, K., et al., *Injection of LPS causes transient suppression of biological clock genes in rats.* J Surg Res, 2008. **145**(1): p. 5-12.
94. Tench, C.M., et al., *The prevalence and associations of fatigue in systemic lupus erythematosus.* Rheumatology (Oxford), 2000. **39**(11): p. 1249-54.
95. Farahani, P., et al., *Community-based evaluation of etanercept in patients with rheumatoid arthritis.* J Rheumatol, 2006. **33**(4): p. 665-70.
96. Grewal, I.S. and R.A. Flavell, *CD40 and CD154 in cell-mediated immunity.* Annu Rev Immunol, 1998. **16**: p. 111-35.
97. Howard, L.M., et al., *Mechanisms of immunotherapeutic intervention by anti-CD40L (CD154) antibody in an animal model of multiple sclerosis.* J Clin Invest, 1999. **103**(2): p. 281-90.
98. Mohan, C., et al., *Interaction between CD40 and its ligand gp39 in the development of murine lupus nephritis.* J Immunol, 1995. **154**(3): p. 1470-80.
99. Furman, R.R., et al., *A phase I study of dacetuzumab (SGN-40, a humanized anti-CD40 monoclonal antibody) in patients with chronic lymphocytic leukemia.* Leuk Lymphoma, 2010. **51**(2): p. 228-35.
100. Schibler, U. and P. Sassone-Corsi, *A web of circadian pacemakers.* Cell, 2002. **111**(7): p. 919-22.
101. Preitner, N., et al., *The orphan nuclear receptor REV-ERBalpha controls circadian transcription within the positive limb of the mammalian circadian oscillator.* Cell, 2002. **110**(2): p. 251-60.
102. O'Neill, L.A. and A.G. Bowie, *The family of five: TIR-domain-containing adaptors in Toll-like receptor signalling.* Nat Rev Immunol, 2007. **7**(5): p. 353-64.
103. Ripperger, J.A. and U. Schibler, *Rhythmic CLOCK-BMAL1 binding to multiple E-box motifs drives circadian Dbp transcription and chromatin transitions.* Nat Genet, 2006. **38**(3): p. 369-74.
104. Adachi, O., et al., *Targeted disruption of the MyD88 gene results in loss of IL-1- and IL-18-mediated function.* Immunity, 1998. **9**(1): p. 143-50.
105. Palin, K., et al., *The type 1 TNF receptor and its associated adapter protein, FAN, are required for TNFalpha-induced sickness behavior.* Psychopharmacology (Berl), 2009. **201**(4): p. 549-56.

106. Teeling, J.L., et al., *Sub-pyrogenic systemic inflammation impacts on brain and behavior, independent of cytokines.* Brain Behav Immun, 2007. **21**(6): p. 836-50.
107. Tollner, B., et al., *The role of tumor necrosis factor (TNF) in the febrile and metabolic responses of rats to intraperitoneal injection of a high dose of lipopolysaccharide.* Pflugers Arch, 2000. **440**(6): p. 925-32.
108. Advani, R., et al., *Phase I study of the humanized anti-CD40 monoclonal antibody dacetuzumab in refractory or recurrent non-Hodgkin's lymphoma.* J Clin Oncol, 2009. **27**(26): p. 4371-7.
109. Hussein, M., et al., *A phase I multidose study of dacetuzumab (SGN-40; humanized anti-CD40 monoclonal antibody) in patients with multiple myeloma.* Haematologica, 2010. **95**(5): p. 845-8.
110. Wierda, W.G., et al., *CD40-ligand (CD154) gene therapy for chronic lymphocytic leukemia.* Blood, 2000. **96**(9): p. 2917-24.
111. Vonderheide, R.H., et al., *Clinical activity and immune modulation in cancer patients treated with CP-870,893, a novel CD40 agonist monoclonal antibody.* J Clin Oncol, 2007. **25**(7): p. 876-83.
112. Spriggs, D.R., et al., *Recombinant human tumor necrosis factor administered as a 24-hour intravenous infusion. A phase I and pharmacologic study.* J Natl Cancer Inst, 1988. **80**(13): p. 1039-44.
113. Sherman, M.L., et al., *Recombinant human tumor necrosis factor administered as a five-day continuous infusion in cancer patients: phase I toxicity and effects on lipid metabolism.* J Clin Oncol, 1988. **6**(2): p. 344-50.
114. Wiley, J.A. and A.G. Harmsen, *Bone marrow-derived cells are required for the induction of a pulmonary inflammatory response mediated by CD40 ligation.* Am J Pathol, 1999. **154**(3): p. 919-26.
115. Kimura, K., et al., *Activated intrahepatic antigen-presenting cells inhibit hepatitis B virus replication in the liver of transgenic mice.* J Immunol, 2002. **169**(9): p. 5188-95.
116. Llovera, M., et al., *Protein turnover in skeletal muscle of tumour-bearing transgenic mice overexpressing the soluble TNF receptor-1.* Cancer Lett, 1998. **130**(1-2): p. 19-27.
117. Saini, A., N. Al-Shanti, and C.E. Stewart, *Waste management - cytokines, growth factors and cachexia.* Cytokine Growth Factor Rev, 2006. **17**(6): p. 475-86.

118. Ryden, M. and P. Arner, *Tumour necrosis factor-alpha in human adipose tissue -- from signalling mechanisms to clinical implications.* J Intern Med, 2007. **262**(4): p. 431-8.
119. Roubenoff, R., et al., *Rheumatoid cachexia: depletion of lean body mass in rheumatoid arthritis. Possible association with tumor necrosis factor.* J Rheumatol, 1992. **19**(10): p. 1505-10.
120. Marcora, S.M., et al., *Randomized phase 2 trial of anti-tumor necrosis factor therapy for cachexia in patients with early rheumatoid arthritis.* Am J Clin Nutr, 2006. **84**(6): p. 1463-72.
121. Metsios, G.S., et al., *Blockade of tumour necrosis factor-alpha in rheumatoid arthritis: effects on components of rheumatoid cachexia.* Rheumatology (Oxford), 2007. **46**(12): p. 1824-7.
122. Fong, Y., et al., *Cachectin/TNF or IL-1 alpha induces cachexia with redistribution of body proteins.* Am J Physiol, 1989. **256**(3 Pt 2): p. R659-65.
123. Zorrilla, E.P., et al., *Interleukin-18 controls energy homeostasis by suppressing appetite and feed efficiency.* Proc Natl Acad Sci U S A, 2007. **104**(26): p. 11097-102.
124. Imeri, L. and M.R. Opp, *How (and why) the immune system makes us sleep.* Nat Rev Neurosci, 2009. **10**(3): p. 199-210.
125. Ripperger, J.A., et al., *CLOCK, an essential pacemaker component, controls expression of the circadian transcription factor DBP.* Genes Dev, 2000. **14**(6): p. 679-89.
126. Petrzilka, S., et al., *Clock gene modulation by TNF-alpha depends on calcium and p38 MAP kinase signaling.* J Biol Rhythms, 2009. **24**(4): p. 283-94.
127. Shang, Y., et al., *Cofactor dynamics and sufficiency in estrogen receptor-regulated transcription.* Cell, 2000. **103**(6): p. 843-52.
128. Vassalli, P., *The Pathophysiology of Tumor Necrosis Factors*. 2005.
129. Hehlgans, T. and K. Pfeffer, *The intriguing biology of the tumour necrosis factor/tumour necrosis factor receptor superfamily: players, rules and the games*, in *Immunology*. 2005. p. 1-20.
130. Pollard, L.C., et al., *Fatigue in rheumatoid arthritis reflects pain, not disease activity.* Rheumatology (Oxford), 2006. **45**(7): p. 885-9.
131. Vgontzas, A.N., et al., *Marked decrease in sleepiness in patients with sleep apnea by etanercept, a tumor necrosis factor-alpha antagonist.* J Clin Endocrinol Metab, 2004. **89**(9): p. 4409-13.

132. Reppert, S.M. and D.R. Weaver, *Coordination of circadian timing in mammals.* Nature, 2002. **418**(6901): p. 935-41.
133. Yamaguchi, S., et al., *The 5' upstream region of mPer1 gene contains two promoters and is responsible for circadian oscillation.* Curr Biol, 2000. **10**(14): p. 873-6.
134. Zheng, B., et al., *Nonredundant roles of the mPer1 and mPer2 genes in the mammalian circadian clock.* Cell, 2001. **105**(5): p. 683-94.
135. Honma, S., et al., *Dec1 and Dec2 are regulators of the mammalian molecular clock.* Nature, 2002. **419**(6909): p. 841-4.
136. Zawel, L., et al., *DEC1 is a downstream target of TGF-beta with sequence-specific transcriptional repressor activities.* Proc Natl Acad Sci U S A, 2002. **99**(5): p. 2848-53.
137. Beg, A.A., et al., *Embryonic lethality and liver degeneration in mice lacking the RelA component of NF-kappa B.* Nature, 1995. **376**(6536): p. 167-70.
138. Rosenfeld, M.E., et al., *Prevention of hepatic apoptosis and embryonic lethality in RelA/TNFR-1 double knockout mice.* Am J Pathol, 2000. **156**(3): p. 997-1007.
139. Balsalobre, A., L. Marcacci, and U. Schibler, *Multiple signaling pathways elicit circadian gene expression in cultured Rat-1 fibroblasts.* Curr Biol, 2000. **10**(20): p. 1291-4.
140. Yamada, K. and K. Miyamoto, *Basic helix-loop-helix transcription factors, BHLHB2 and BHLHB3; their gene expressions are regulated by multiple extracellular stimuli.* Front Biosci, 2005. **10**: p. 3151-71.
141. Krippner-Heidenreich, A., et al., *Control of receptor-induced signaling complex formation by the kinetics of ligand/receptor interaction.* J Biol Chem, 2002. **277**(46): p. 44155-63.
142. O'Neill, J.S., et al., *cAMP-dependent signaling as a core component of the mammalian circadian pacemaker.* Science, 2008. **320**(5878): p. 949-53.
143. Harrisingh, M.C., et al., *Intracellular Ca2+ regulates free-running circadian clock oscillation in vivo.* J Neurosci, 2007. **27**(46): p. 12489-99.
144. Ikeda, M., et al., *Circadian dynamics of cytosolic and nuclear Ca2+ in single suprachiasmatic nucleus neurons.* Neuron, 2003. **38**(2): p. 253-63.
145. Akiyama, M., et al., *Inhibition of light- or glutamate-induced mPer1 expression represses the phase shifts into the mouse circadian locomotor and suprachiasmatic firing rhythms.* J Neurosci, 1999. **19**(3): p. 1115-21.

146. Tischkau, S.A., et al., *Ca2+/cAMP response element-binding protein (CREB)-dependent activation of Per1 is required for light-induced signaling in the suprachiasmatic nucleus circadian clock.* J Biol Chem, 2003. **278**(2): p. 718-23.
147. Zawel, L., et al., *DEC1 is a downstream target of TGF-beta with sequence-specific transcriptional repressor activities*, in *Proc Natl Acad Sci USA*. 2002. p. 2848-53.
148. Buerki, C., et al., *Functional relevance of novel p300-mediated lysine 314 and 315 acetylation of RelA/p65.* Nucleic Acids Res, 2008. **36**(5): p. 1665-80.
149. Schagger, H., *Tricine-SDS-PAGE.* Nat Protoc, 2006. **1**(1): p. 16-22.
150. Okun, M.L., et al., *Exploring the cytokine and endocrine involvement in narcolepsy.* Brain Behav Immun, 2004. **18**(4): p. 326-32.
151. Vgontzas, A.N. and G.P. Chrousos, *Sleep, the hypothalamic-pituitary-adrenal axis, and cytokines: multiple interactions and disturbances in sleep disorders.* Endocrinol Metab Clin North Am, 2002. **31**(1): p. 15-36.
152. Siegel, J.M., *Sleep viewed as a state of adaptive inactivity.* Nat Rev Neurosci, 2009. **10**(10): p. 747-53.
153. Sharma, R. and S.D. Anker, *Cytokines, apoptosis and cachexia: the potential for TNF antagonism.* Int J Cardiol, 2002. **85**(1): p. 161-71.
154. Gao, B., *Cytokines, STATs and liver disease.* Cell Mol Immunol, 2005. **2**(2): p. 92-100.
155. Konsman, J.P., et al., *The vagus nerve mediates behavioural depression, but not fever, in response to peripheral immune signals; a functional anatomical analysis.* Eur J Neurosci, 2000. **12**(12): p. 4434-46.
156. Hanisch, U.K., *Microglia as a source and target of cytokines.* Glia, 2002. **40**(2): p. 140-55.
157. Bette, M., et al., *Constitutive expression of p55TNFR mRNA and mitogen-specific up-regulation of TNF alpha and p75TNFR mRNA in mouse brain.* J Comp Neurol, 2003. **465**(3): p. 417-30.
158. Nadeau, S. and S. Rivest, *Effects of circulating tumor necrosis factor on the neuronal activity and expression of the genes encoding the tumor necrosis factor receptors (p55 and p75) in the rat brain: a view from the blood-brain barrier.* Neuroscience, 1999. **93**(4): p. 1449-64.
159. Farrar, W.L., et al., *Visualization and characterization of interleukin 1 receptors in brain.* J Immunol, 1987. **139**(2): p. 459-63.

160. Yabuuchi, K., et al., *Localization of type I interleukin-1 receptor mRNA in the rat brain.* Brain Res Mol Brain Res, 1994. **27**(1): p. 27-36.
161. Panda, S., et al., *Coordinated transcription of key pathways in the mouse by the circadian clock.* Cell, 2002. **109**(3): p. 307-20.
162. Nakahata, Y., et al., *Circadian control of the NAD+ salvage pathway by CLOCK-SIRT1.* science, 2009. **324**(5927): p. 654-7.
163. Sahar, S. and P. Sassone-Corsi, *Metabolism and cancer: the circadian clock connection.* Nat Rev Cancer, 2009. **9**(12): p. 886-96.
164. Rutter, J., et al., *Regulation of clock and NPAS2 DNA binding by the redox state of NAD cofactors.* science, 2001. **293**(5529): p. 510-4.
165. Yazdanpanah, B., et al., *Riboflavin kinase couples TNF receptor 1 to NADPH oxidase.* Nature, 2009. **460**(7259): p. 1159-63.

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### Publications

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